The role of mTOR signalling pathway as a susceptibility factor in genotoxic stress-induced cell death

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Submitted to the faculty of Medicine,

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by M.Sc., Mangalore Govind Pai

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List of abbreviations

Aa	Essential amino acids
2dG	2-deoxy-Glucose
4E-BP1	Eukaryotic translation initiation factor 4E binding protein 1
TOP	5'-tract of oligo-pyrimidine
PRPP	5-phosphoribosyl-1-pyrophosphate
ADHD	Attention Deficit Hyperactivity Disorder
ADP	Adenosine diphosphate
Adr	Adriamycin
AMPK	AMP-activated kinase
ANOVA	Analysis of variance
Chk1	Checkpoint kinase 1
ATP	Adenosine triphosphate
ATM	Ataxia telangiectasia mutated
ATR	ATM and RAD3-related
ATG139	Autophagy related gene–13
CAD	Carbamoyl-phosphate synthetase 2, Aspartate transcarbamylase, and Dihydroorotase
CLIP1	CAP-GLY domain containing linker protein 1
CFSE	Carboxyfluorescein succinimidyl ester
Cdk	cyclin-dependent kinase
DAPI	4',6-Diamidino-2-phenylindole
DDR	DNA damage response
DAPK1	Death-associated protein kinase 1
E2F	E2F family of transcription factors
EdU	5–Ethynyl–2'–deoxyUridine
ERK	Extracellular signal-Regulated Kinase
ER-stress	Endoplasmic Reticulum stress

FACS	Fluorescence-Activated Cell Sorting
FKBP12	FK506 binding protein of 12 kDa
FRB	FK506-Rapamycin-Binding
GEF	Guanine nucleotide Exchange Factor
GF	Growth Factor
GLc	D-Glucose
GAP	GTPase-Activating Protein
HU	Hydroxyurea
IIS/TOR	Insulin or IGF signalling and Target of Rapamycin
IFNγ	Interferon gamma
IGF	Insulin-like Growth Factor
IRS1	Insulin Receptor Substrate 1
L-Gln	L-Glutamine
LPIN1	Lipin1
LKB1	Liver Kinase B1
LAM	Lymphangiomyomatosis
MMS	Methyl Methane Sulphonate
MRN complex	Mre11-Rad50-Nbs1 Complex
mTOR	mammalian or mechanistic Target of Rapamycin
mTORC2	mTOR, complex 2
mTORC1	mTOR, complex 1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
Nbs1	Nijmegen Breakage Syndrome 1
NF1	Neurofibromin-1
Nsd	Nucleosides
PARP	Poly ADP–Ribose Polymerase
PGC-1α	Peroxisome proliferator-activated receptor gamma co-activator 1-alpha
PI	Propidium Iodide

PI3K	Phosphoinositide-3-kinase
PTEN	Phosphatase and Tensin homologue
pThr14 cdk2	inhibitory phosphorylation at thr14 of cdk2
Rap	Rapamycin
RS	Replication stress / S-phase stress
Rb	Retinoblastoma protein
RS-like	phenotype, replication stress–like
S6K	S6 kinase
SD	Standard Deviation
Sesn1	Sestrin1
Sesn2	Sestrin1
ssDNA	Single-stranded DNA
SREBP1	Sterol Regulatory Element-Binding Protein 1
STS	Staurosporine
SEGAs	Sub-Ependymal Giant cell Astrocytomas
SENs	Sub-Ependymal Nodules
Torin1	ATP competitive TOR Kinase inhibitor,
TSC	Tuberous Sclerosis Complex
TSC1-/-	Hamartin null (knockout)
MEFs	Mouse Embryo Fibroblast
ULK1	Unc-51 like autophagy activating kinase 1
UPR	Unfolded Protein Response
v-ATPase	vacuolar ATP hydrolase
WT	wild type
WT MEFs	Hamartin Wild-type (TSC1+/+) Mouse Embryonic Fibroblasts

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Summary

Tuberous Sclerosis (TS) resulting from high mTORC1 activity upon mutational loss of TSC1/2 is characterised by multiple benign tumours. Loss of TSC function, as opposed to other tumours driven by upstream mutations affecting the very pathway, results in the manifestation of highly apoptotic, non-malignant neoplasias. Contradictory to its established growth-promoting role in cancers with modest mTOR activation, exorbitantly high mTOR activity in TSC-/- cells can sensitise them to stress-induced cell-cycle arrest and/or apoptosis. Seminal reports identified that TSC-/- cells are not only maintained in a benign state but also susceptible to sub-lethal genotoxic stress, at least in part by increased p53 function. Studies thereafter unveiling energetic shortfall and a metabolic collapse, due to increased anabolic demand and lack of resource-sensing (upstream of TSC in TSC-/- cells) respectively, refined our understanding of the stress-sensitivity phenomenon. However, little is known about how cell cycle irregularities and altered progression through the S-phase addressed over two decades renders TSC-/- cells hypersensitive to sub-lethal genotoxic stress.

We have observed higher DNA damage accrual and an exacerbated death toll in TSC1-/- MEF cells in presence of mild, acute doses of single or double strand break-inducing agents. Multi-colour flow cytometry revealed an altered cell cycle distribution accompanied by elevated overall nucleotide-incorporation rates suggesting inherent replication stress (RS), frail cell cycle regulation and dampened checkpoint responses. DNA fibre assays for S-Phase dynamics indicated that constitutive mTORC1 activity in TSC1-/- MEFs drives a subtler replication stress-like (RS-like) phenotype characterized by modest over-firing of replication origins, slower-progressing, symmetric forks with elevated genotoxin-induced asymmetry thus sensitizing them. Despite RS- and G2/M-checkpoint signalling proficiency, incessant S-phase progression under genotoxic stimuli accompanied by an apparently leaky damage-prone mitotic entry, raises the odds of mitotic catastrophe even though genomic instability has not been documented in TS. Moreover, cellular energetic status seems to augment and synergise with the effect of mild external genotoxic stress on TSC1-/- cells. Our data suggest that constitutive mTORC1 activity, by promoting non-canonical replication stress, causing energetic debility and DNA damage checkpoint override, proves detrimental to cellular

stress adaptation. Importantly, our interpretation of the spontaneous asymmetry-free S-phase, a deviation from classical RS, provides novel insight as to why TSC tumours are detained in a benign state and opens avenues for low-dose chemotherapy-based therapeutic intervention in TSC patients.

Key words: Tuberous Sclerosis, mTORC1, Replication Stress, Genotoxic stress, hormesis.

Zusammenfassung

Die tuberöse Sklerose (TS) resultiert aus einer erhöhten mTORC1 Aktivität aufgrund des TSC1/2 Mutationsverlustes und ist durch meist gutartige Tumore gekennzeichnet. Der TSC-Funktionsverlust resultiert, im Gegensatz zu anderen Tumoren, welche durch vorgeschaltete Mutationen den gleichen Signalweg beeinflussen, in der Manifestierung von hoch-apoptotischen, nicht-malignen Neoplasien. Widersprüchlich zu der etablierten wachstumsfördernden Rolle, welche geringe mTOR-Aktivität bei Krebserkrankungen aufweist, kann exorbitant hohe mTOR Aktivität in TSC-/- Zellen gegen Stress-induzierten Zellzyklus-Arrest und/oder Apoptose sensibilisieren. Frühere Studien stellten fest, dass TSC-/- Zellen nicht nur in einem gutartigen Zustand gehalten werden, sondern auch anfällig für subletalen genotoxischen Stress sind, welcher zumindest teilweise durch eine erhöhte p53-Funktion vermittelt wird. Darauffolgende Studien enthüllten energetische Defizite und einen Zusammenbruch des Stoffwechsels, ausgelöst durch erhöhten anabolischen Bedarf und fehlendes Erfassen der Ressourcen (vorangelagert von TSC in TSC-/- Zellen) und konkretisierten unser Verständnis des Stress-Empfindlichkeits-Phänomens. Wenig ist jedoch darüber bekannt, wie die Zellzyklusunregelmäßigkeiten und der veränderte Verlauf der S-Phase, welche seit mehr als zwei Jahrzehnten untersucht werden, TSC-/- Zellen überempfindlich gegen subletalen genotoxischen Stress macht. Wir haben vermehrte Akkumulation von DNA-Schäden und eine erhöhte Sterberate in TSC1-/- MEF-Zellen in Anwesenheit von milden Dosen Einzel- oder Doppelstrangbruch-verursachender Agenzien beobachtet. Mehrfarben-Durchflusszytometrie zeigte eine veränderte Zellzyklus-Verteilung, begleitet von erhöhten Gesamt-Nukleotid-Einbauraten, welche auf dazugehörigen Replikations-Stress (RS), mangelnde Regulation des Zellzyklus und verminderte Kontrollpunkt-Reaktionen hindeuten. DNA-Faser-Assays zur Analyse der S-Phasen Dynamik zeigten, dass dauerhafte mTORC1 Aktivität in TSC1-/- MEFs zu einem milderen Replikations-Stress-ähnlichen (RS-ähnlich) Phänotyp, gekennzeichnet durch geringes Überfeuern der Replikationsursprünge, jedoch deutlich langsameres Fortschreiten und symmetrische Replikations-Gabeln mit erhöhter genotoxisch-induzierter Asymmetrie führt und diese dadurch sensibilisiert. Trotz RS- und G2/M-Kontrollpunkt Signalübertragungsfähigkeit, erhöht die voranschreitende S-Phasen-Progression unter genotoxischer Stimulation, begleitet von offensichtlich fehlerhaftem, schadensanfälligen

Mitosen-Eintritt, die Wahrscheinlichkeit einer mitotischen Katastrophe, obwohl genomische Instabilität nicht in TS dokumentiert ist.

Des Weiteren scheint der zelluläre Energiestatus den Effekt des milden externen genotoxischen Stresses auf die TSC1 -/- Zellen zu verstärken und einen synergistischen Effekt zu erzielen. Unsere Daten legen nahe, dass die dauerhafte mTORC1 Aktivität, welche energetische Schwäche und das Übergehen der DNA-Schäden-Kontrollpunkte verursacht, ausgelöst durch die Förderungen von nicht-kanonischem Replikations-Stress, sich als schädlich für die zelluläre Stress-Adaptation erweist. Am wichtigsten aber ist, dass unsere Interpretation der spontanen Asymmetrie-freien S-Phase, welche eine Abweichung von dem klassischen RS darstellt, neue Einblicke ermöglicht, warum TSC Tumore in einem gutartigen Zustand verbleiben und öffnet Wege für eine niedrig-dosierte Chemotherapie-basierende therapeutische Behandlung bei TSC-Patienten.

1. Introduction

1.1. Growth signalling and cancer

Eukaryotic cells coordinate cellular growth with nutrient availability and environmental favourability. Tumorigenesis entails a complex sequence of events leading to the loss of several mechanisms regulating cell growth and proliferation in response to extrinsic cues including growth factor stimuli and a conducive, stress-free environment. Insights from over three decades of fundamental cancer research have revealed that mutations in signalling pathway players controlling cell growth drive tumorigenesis in higher eukaryotes including mammals and man. The Ras, PI3K (Phosphoinositide-3-kinase) and mTOR (mammalian or mechanistic target of rapamycin) signalling pathways, three prominent and intricately linked cellular growth control pathways when mutated, drive cell growth unconstrained by environmental inputs. It is noteworthy that most human tumours harbour activating mutations of Ras and PI3K or inactivating mutations in negative regulators of these proteins such as PTEN (phosphatase and tensin homologue) or NF1 (neurofibromin-1). The mTOR protein kinase has emerged as a critical growth-control hub, receiving stimulatory signals from Ras and PI3K upon growth factor stimulation, as well as nutrient availability (amino-acids, glucose and oxygen). The pre-eminence of mutations observed in these interconnected pathways suggests that defeating growth-control checkpoints and promoting cell survival in undernourished conditions may be a primeval event in tumorigenesis. Eventually, these pathways promote tumorigenesis and foster tumour growth through the coordinated phosphorylation of proteins that regulate protein synthesis, cell-cycle progression and metabolism, and the transcription of genes controlling these processes (Hanahan & Weinberg, 2000; Martin, 2003; Shaw & Cantley, 2006).

In this piece of work, we highlight mTORC1 (mechanistic Target of Rapamycin, complex 1) as an important mediator of signalling in response to metabolic and genotoxic stress stimuli and sought out to investigate how cell cycle control by mTORC1 influences cellular genotoxic stress response. Based on our findings, we also discuss issues inherent to tuberous sclerosis, a non-malignant tumour syndrome resulting from constitutive activity of the mTOR pathway, that compromise cellular adaptation to genotoxic stress, also reasoning why tumours characteristic of TSC remain benign by the very virtue of their genotype/nature.

1.2. mTOR signalling pathway

mTOR (mechanistic Target of Rapamycin) is a highly conserved Ser/Thr kinase belonging to the PIKK (PI3 kinase- related kinases) family. mTOR forms two structurally and functionally distinct multiprotein complexes, mTORC1 (complex 1) and mTORC2 (complex 2), which correspond to two major branches within the overall signalling network. Both mTORC1 and mTORC2 are atypical serine/threonine kinases. Best studied for its role in translation master-control and cell growth, mTORC1 integrates a variety of extrinsic and intracellular inputs, including growth factors (insulin), nutrients (amino acids), cellular energy status and stress, to regulate several anabolic and catabolic processes. The tumour suppressor complex TSC1-TSC2 (Hamartin–Tuberin, collectively TSC) is a signalling nexus that negatively regulates mTORC1 activity by functioning as a GTPase-activating protein (GAP) for Rheb.

In response to growth factors, AKT or Rsk phosphorylates and inactivates TSC1-TSC2, allowing GTP-bound Rheb (a Ras-like GTPase) to activate mTORC1. Low energy (cellular energy status) inhibits mTORC1 by activating AMPK, which phosphorylates and activates TSC1-TSC2. Although the pathway by which amino acids, the branched chain amino acid (BCAA) leucine in particular, activate mTORC1 is poorly understood, mTORC1 activation is bipartite. In a first step, intracellular amino acid pools promote its translocation from the cytosol to the lysosomal surface. By an incompletely understood mechanism, amino acid sensing from within the lysosomal lumen by a v-ATPase-requiring process, initiates the GEF activity of the Ragulator complex toward RagA within the heterodimeric Rag GTPases. Upon GTP binding, RagA recruits mTORC1 to the lysosomal surface, allowing it to interact with the small GTPase Rheb, a potent stimulator of mTORC1 kinase activity. Upon growth factor stimulation, nucleotide loading and lysosomal membrane localisation of the small GTPase Rheb is regulated by TSC in a subsequent step leading to the activation of mTORC1 (Bar-Peled & Sabatini, 2014; Dibble & Manning, 2013; Groenewoud & Zwartkruis, 2013; Menon et al., 2014).

Many environmental and intracellular cues that impinge on mTORC1 funnel through TSC and regulate its GAP activity toward Rheb. In addition to growth-factor dependent Akt-mediated mTORC1 activation, ERK also imposes positive control on the pathway by directly phosphorylating ser664 of TSC2 to negatively regulate its function. AMPK activation, either by LKB1 under low cellular energy levels (low ATP to ADP ratios) or in a p53-dependent

manner following DNA damage, leads to phosphorylation and activation of TSC, thus inactivating mTORC1. Hypoxia induces Redd1 expression, which by an ill-defined process maintains TSC function (Feng, 2010; Feng, Zhang, Levine, & Jin, 2005; Ken. Inoki, Zhu, & Guan, 2003; R. G. Jones et al., 2005; Laplante & Sabatini, 2012).

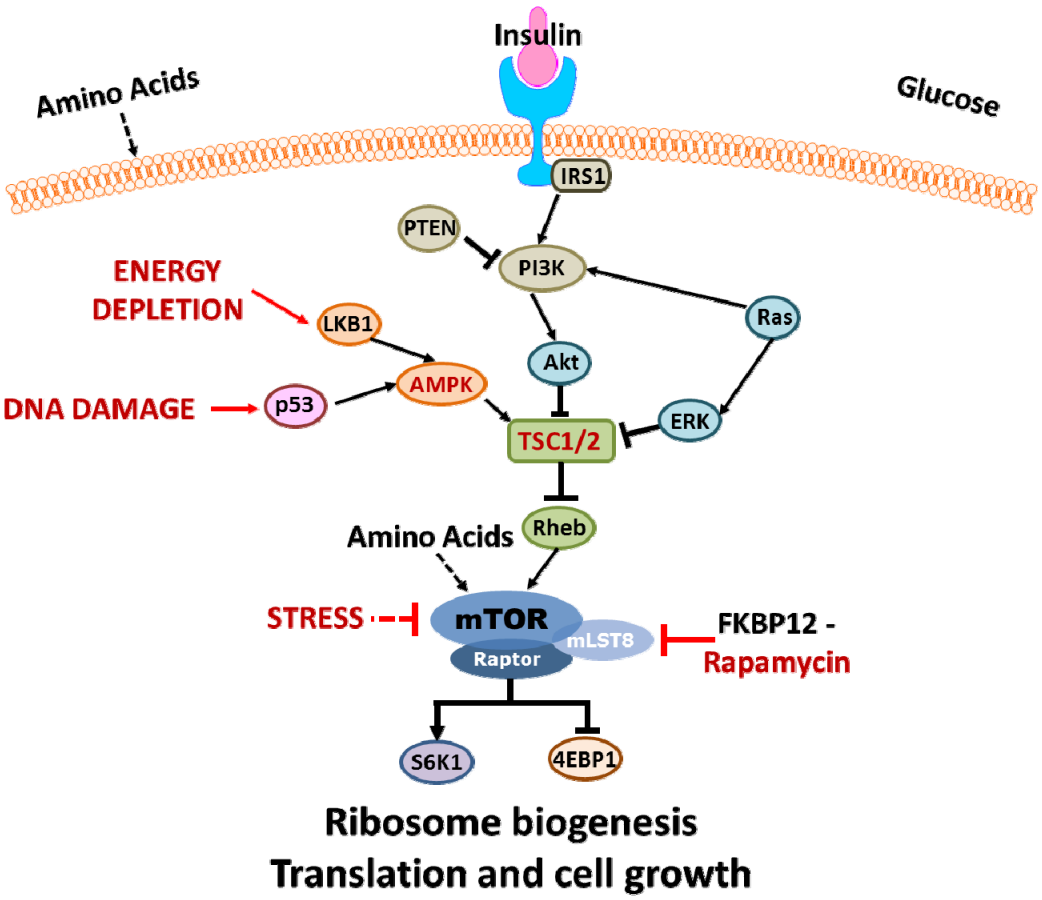


Figure 1: Simplified scheme of the mTOR pathway
The figure portrays mTORC1 as an integrator of diverse inputs regulating cell size, metabolism and growth. In a multi-step activation process, mTORC1 integrates extraneous and intrinsic stimuli viz., growth factors and nutrient availability; under favourable conditions in the absence of stress, fuels cell growth by directly controlling protein synthesis. Rapamycin, a macrolide antibiotic, is able to selectively inhibit mTORC1 in a specific complex with FKBP12 by allosteric mechanisms, resulting in complete inactivation of its effector substrate S6 Kinase, while only partially inhibiting 4EBP1 depending on the cell-type.

The downstream targets of mTORC1 pathway encompass a range of anabolic and catabolic programmes including protein synthesis, ribosome biogenesis, lipid biosynthesis, macro-autophagy, and transcription of biosynthetic pathway genes. mTOR is known to directly or indirectly regulate phosphorylation of about 800 protein substrates. Once activated, mTORC1 enables growth by promoting anabolic programmes while repressing

catabolism. mTORC1 controls cap-dependent 5'-tract of oligo-pyrimidine (TOP) mRNA translation by phosphorylation-mediated activation of S6 kinase (S6K) and inactivation of translation repressor 4E-BP1 (Acosta-Jaquez et al., 2009; Fingar & Blenis, 2004; Fingar et al., 2003; Zoncu, Efeyan, & Sabatini, 2011) and also regulates lipid biosynthesis through sterol regulatory element-binding protein 1 (SREBP1) and Lipin1 (LPIN1) (Bakan & Laplante, 2012). mTORC1 plays an important role in maintaining energy homeostasis. It regulates mitochondrial biogenesis through peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) (Laplante & Sabatini, 2013) and restrains autophagy by inactivating autophagy related gene–13 (ATG139), Unc-51 like autophagy activating kinase 1 (ULK1), and Death-associated protein kinase 1 (DAPK1) (J. Kim, Kundu, Viollet, & Guan, 2011; Lamb, Yoshimori, & Tooze, 2013). mTORC1 stimulates *de novo* purine and pyrimidine biosynthesis through S6K–mediated phosphorylation of the multifunctional biosynthetic enzyme complex CAD and transcriptional enhancement of the pentose phosphate pathway which produces 5-phosphoribosyl-1-pyrophosphate (PRPP), an allosteric activator of CAD (Ben-Sahra, Howell, Asara, & Manning, 2013; Buel, Kim, & Blenis, 2013). Among other potential targets mTORC1 may phosphorylate CAP-GLY domain containing linker protein 1 (CLIP1) and regulate microtubules. S6K in addition to phosphorylating various translational targets also phosphorylates to feedback on insulin receptor substrate 1 (IRS-1) for growth factor signal attenuation.

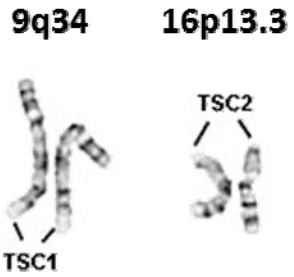
The immunosuppressant rapamycin in complex with FKBP12 binds to specifically inhibits mTORC1 but not mTORC2 (Eric J. Brown et al., 1994). Until recently, most previous work in mammalian systems focused on the rapamycin-sensitive mTORC1 complex (Ballou & Lin, 2008; Beauchamp & Platanias, 2013). The relatively understudied mTORC2 promotes cell survival through direct phosphorylation of ser473 in the hydrophobic motif of AKT (Cybulski & Hall, 2009; Yang & Guan, 2007).

At the organismal level, dysregulation of the mTORC1 pathway is prevalent in several human pathologies, given its role as a master regulator of cell metabolism. Cancers with aberrant mTORC1 activity, such as tuberous sclerosis and advanced renal cell carcinoma, are increasingly treated with Rapamycin analogues (Rapalogs). Furthermore, hyper-activation of this pathway leads to the down-regulation of IRS1 and progression of type 2 diabetes (Dann, Selvaraj, & Thomas, 2007). mTOR also mediates nutrient-related processes such as appetite

control and aging. Although the mTORC1 pathway is indispensable to mammalian development, reduction of mTORC1 activity in mice models through pharmacological inhibition not only enhances adult stem cell numbers, function, or both, but also extends murine life span (Lamming, Ye, Sabatini, & Baur, 2013). Hence, mTORC1, by integrating extracellular inputs and governing cell growth, lies at the crux of cellular physiology and homeostasis.

1.3.Tuberous Sclerosis Complex (TSC)

Tuberous Sclerosis (TSC) results from high mTORC1 activity upon mutational loss of TSC1/TSC2 and is characterised by multiple benign tumours. TSC is a multi-organ disorder characterized by tumour-like lesions called hamartomas in the brain, skin, heart, kidneys and lungs. TSC manifests with a broad range of clinical features with multisystem involvement from childhood through adult life. Although an accurate ascertainment is difficult, from available statistics, the birth incidence or childhood prevalence is estimated to be in the order of 1 in 6000 live births. TSC children present with epilepsy (80%) and mental retardation (44%). Childhood seizures, infantile spasms in particular, behavioural anomalies including autism and attention deficit hyperactivity disorder (ADHD), and sleep disturbances are among the most common presentations, with varying seizure patterns evolving through childhood. Cranial imaging reveals abnormalities in 90-95% of all TSC patients, and common brain lesions include cortical tubers, sub-ependymal nodules and sub-ependymal giant cell astrocytomas (SEGAs). Mental retardation seems to be proportional to the extent of brain manifestations, even among patients of the same age. Dermatological and cutaneous manifestations occupy an important place in the diagnostic criteria for TSC and include hypo-pigmented macules in infancy, facial angiofibromas particularly on the nose, cheeks and chin around 5 years of age, Fibrous plaques on the forehead, Shagreen patches or connective tissue *naevi* on the lower back during childhood, and ungual fibromas of the finger and toe nails in adolescence and adulthood. Cardiac rhabdomyomas, renal angiomyolipomas and pulmonary lymphangiomyomatosis (LAM) mark manifestations of the heart kidneys and lungs respectively. Although life expectancy in general is normal in TSC



The TSC1 and TSC2 loci on human chromosomes 9 and 16 respectively.
Source: Tuberöse Sklerose Deutschland e. V.

patients if treated and managed well, it largely depends on the extent of brain and kidney manifestations as SEGAs and LAMs pose as serious health complications. Gingival fibromas are also observed. A detailed account of the clinical manifestations in TSC is available from several online resources and some of the most common ones are depicted here (Napolioni & Curatolo, 2008; Shepherd, Gomez, Lie, & Crowson, 1991; *Tuberous Sclerosis Complex Genes Clinical Features and Therapeutics*, 2010; Yates, 2006).

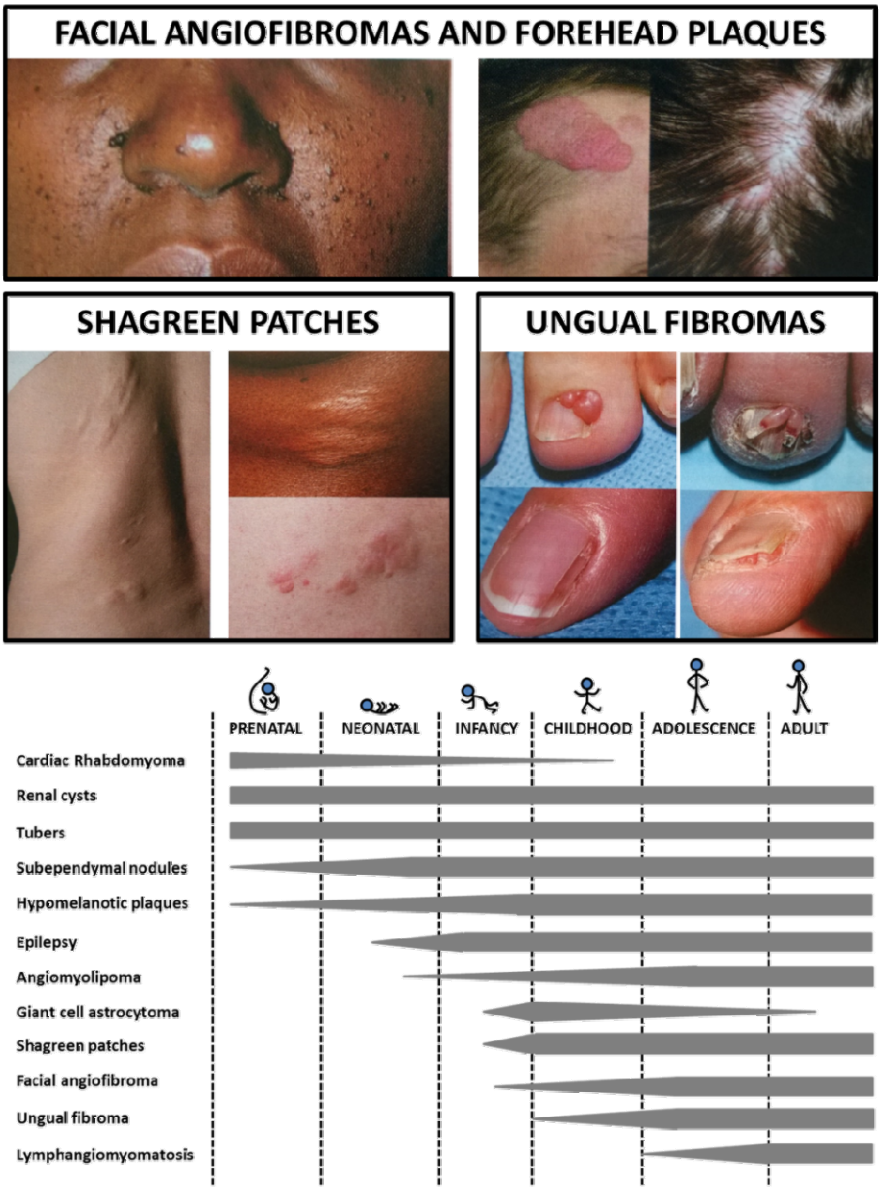


Figure 2: Common symptoms and features of tuberous sclerosis complex
Above: Common symptoms of Tuberous Sclerosis. Excerpts reproduced with permission from Tuberous Sclerosis Complex: Genes, Clinical Features and therapeutics, Wiley-Blackwell, 2010, edited by David J Kwiatkowski, Vicky Holets Whittemore, and Elizabeth A. Thiele, pp. 288-291.
Below: Milestones of symptom-development in tuberous sclerosis patients. Translated and modified with permission from Schweiz Med Forum 2013;13(36), page 697.

Tuberous Sclerosis complex is an inherited, autosomal dominant syndrome and is caused by inactivating mutations in either TSC1 (Hamartin) or TSC2 (Tuberin) genes, which cooperate in a functional heterodimeric complex – TSC, to negatively regulate the mTOR complex 1 (Zhang et al., 2003). Both TSC1 and TSC2 are relatively large genes mapped to the loci 9q34.13 and 16p13.3 respectively in humans. About one-third of all cases have an inherited defect in either TSC1 or TSC2 and the remaining two-thirds of the cases appear to be sporadic with *de novo* mutations. TSC1 mutations are more common in familial cases while sporadic cases are predominantly TSC2 mutations. The mutation spectrum of TSC1 and TSC2 is tremendously diverse and disease severity is highly variable, even within families. Over 450 disease-causing mutations in TSC1 and over 1300 for TSC2 have been reported, although the mutations are varied and largely span the entire length of the genes with no identified hot-spots. Additional information including identified mutations, published or otherwise, are indexed in several *Tuberous sclerosis databases*
[http://www.tuberous-sclerosis.org/,
http://www.tsalliance.org/,
http://www.tsdev.org/deutsch/willkommen/-/181,181,92001,liste9.html].
Mutation screening is expensive since sequencing approaches are alone valid for detection of large deletions, small insertions and point mutations. Available data point out that sporadic TSC2 mutations manifest a more severe phenotype compared to TSC1 sporadic cases. On the other hand, certain missense mutations and mosaicism in TSC2 cases are known to be associated with a particularly mild disease phenotype. In addition to variation in severity attributed to differences between TSC1 and TSC2 mutations, variability also comes from somatic mosaicism and the influence of modifier genes for e.g., higher interferon gamma (IFNγ) expression and lower renal angiomyolipomas frequencies in TSC2 patients (K. Inoki & Guan, 2009; Mayer, Fonatsch, Wimmer, van den Ouweland, & Maat-Kievit, 2014; *Tuberous Sclerosis Complex Genes Clinical Features and Therapeutics*, 2010; Yates, 2006)

Table 1: Summary of structural organisation and mutation spectrum of human tuberous sclerosis genes

	TSC1	TSC2
Locus	9q34.13	16p13.3
Gene span	55 kb	44 kb
Exons	23	42
Transcript	8.6 kb 21 coding exons Exons 1 and 2 – 5' UTRs 3.5 kb coding region	5.5 kb 41 coding exons Exons 25 and 31 alternatively spliced 5.4 kb coding region
Protein	**Hamartin** 1164 amino acids 130 kDa N-term. Transmembrane domain C-term. Coiled coil domain	**Tuberin** 1784 amino acids 180 kDa Potential catalytic domain at C-term. RAP1GAP homology
Mutation rate	12 – 19 %	65 – 70 %
Mutation sites	Entire gene. No apparent hotspots.	Entire gene. No apparent hotspots.
Mutation type	Inactivating, mostly nonsense, frame-shift and splice-site mutations.	Inactivating, including missense, in-frame and large deletions/rearrangements involving adjacent PKD1 locus.

1.4. mTORC1 in metabolic diseases and Cancer – Tuberous Sclerosis is a unique genetic syndrome

Tuberous Sclerosis results from high mTORC1 activity upon mutational loss of TSC1/TSC2 and is characterised by multiple benign tumours. Several established and recently identified tumour suppressors including LKB1, AMP-responsive protein kinase–AMPK (under energy scarcity) and PTEN, NF1 (negative regulators of PI3K and Ras respectively) that attenuate mTOR signalling under unfavourable conditions, are inactivated in familial human tumour syndromes. Some of these share clinical features collectively termed Phakomatoses (Korf, 2005; Lin & Barker, 2006; Nowak, 2007). The tumour suppressor complex–TSC is a signalling node that integrates these upstream inputs to ensure cell proliferation strictly under favourable environmental conditions (Hengstschläger et al., 2001; Rosner, Hofer, Kubista, & Hengstschlager, 2003). Loss of TSC function is unique in that, as opposed to other tumours driven by upstream mutations affecting the same pathway, results in the manifestation of highly apoptotic, non-malignant neoplasias. In addition to such phenotypic deviations from other cancers featuring high mTORC1 activity i.e., benign manifestation and high apoptotic

tendency in TSC hamartomas, even at the cellular level TSC represents a unique single-gene disorder in which the loss of one gene (TSC1 or TSC2) results in diverse metabolic and molecular outcomes. Firstly, cells lack resource/nutrient sensing mechanisms upstream and their growth is somewhat growth factor independent following TSC loss. Several complications are apparent downstream of TSC, including sustained protein synthesis resulting in increased cell mass/growth, endoplasmic reticulum overload and ER-stress accompanied by UPR initiation (Li et al., 2015; Zhou et al., 2009) and an increased, but unmet energy demand due to a hyper-anabolic state of these cells (R. T. Abraham & Eng, 2010; Chiu et al., 2012). Consequently, persistent feedback inhibition of IRS1 by S6K also results in insulin resistance (Hunter & Shah, 2005; Shah, Wang, & Hunter, 2004; Um, D'Alessio, & Thomas, 2006). Transcriptional and translational changes additionally influence cell cycle progression in TSC cells (refer to section mTORC1 in cell cycle control)

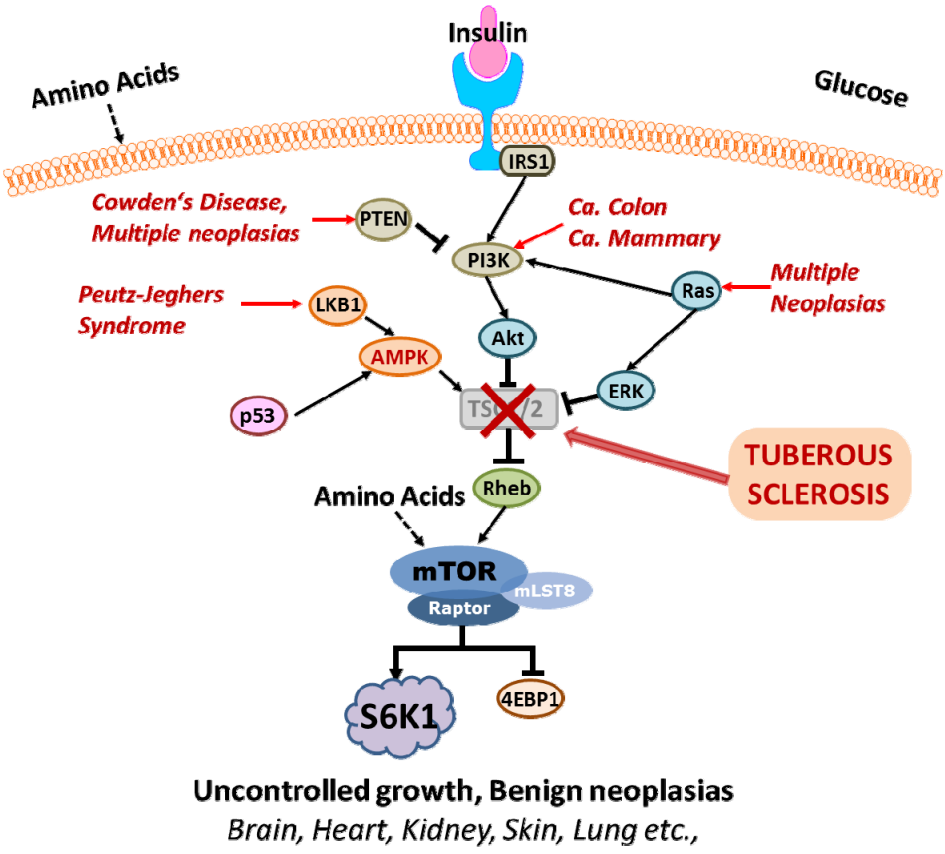


Figure 3: Tuberous sclerosis is distinct from other tumours
Several upstream mutations in known oncogenes or tumour suppressors driving aggressive metastatic cancers funnel signals via TSC, resulting in modest mTORC1 hyperactivity; however, loss of TSC function represents superlative mTORC1 activation states while manifesting as benign tumours with apoptotic tendency.

In marked opposition to its well established growth-promoting role in cancers with moderately high mTOR activation, exorbitantly high mTOR activity in TSC-/- cells can sensitise them to metabolic and genotoxic stress-induced cell-cycle arrest and/or apoptosis. Previous studies have identified that TSC-/- cells are not only maintained in a benign state but also susceptible to sub-lethal genotoxic stress, at least in part by increased p53 function, including translational activation of pro-apoptotic and anti-proliferative factors (Lee et al., 2007). Although metabolic stress is malefic for TSC-/- cell survival, only recently was energetic insufficiency due to a demand–supply gap and a metabolic collapse attributed to their heightened stress sensitivity (Choo et al., 2010; S. G. Kim et al., 2013). Our own findings reveal L-glutamine as a sustained, anaplerotic energy source in line with other reports (Csibi et al., 2013; Durán & Hall, 2012; Duran et al., 2012), while essential amino acids seem to accelerate cell death in TSC1-/- cells, possibly by driving translation and augmenting ER-stress, in the absence of glucose. We and others have also observed a particularly strong death outcome in TSC1-/- cells subjected to mild, acute doses of genotoxins. Cell cycle disturbances, including imbalances in G1/S phase Cyclins and Cdk inhibitor p27 expression (T. Soucek, Yeung, & Hengstschlager, 1998), stunted G1 phase and a prolonged S phase (Wataya-Kaneda et al., 2001) have been described as characteristics of TSC-/- cells with constitutive mTORC1 activity. However, the nature of these deleterious cell cycle alterations and whether this has implications on their sensitivity to sub-lethal genotoxic stress remains largely unknown. In this piece of work, we wish to investigate mechanisms contributing to the loss of adaptive capacity in TSC-/- cells resulting in such hormetic death outcome to sub-lethal genotoxic stress.

Several open questions remain to be addressed, for e.g., how is the cell cycle of TSC-/- cells in totality, affected by such perturbations in cell cycle regulatory proteins? Does that yield insights into why TSC-/- cells are maintained in a benign state? What predisposes these robustly growing cells to mild stress-induced death and how does low energy status influence this? Does inhibition of the mTOR pathway restore phenotypes?

1.5. mTORC1 and cancer therapy.

The serine/threonine kinase mTOR regulates fundamental aspects like cell size, transcription, translation, autophagy, cell survival and ageing. Obesity, diabetes, cancer and degenerative diseases are among some whose genesis involves dysregulation of mTOR pathway, provoking fierce interest from varied therapeutic areas (Rosner & Hengstschlager, 2011). 80% of all cancers directly or indirectly acquire mTOR hyperactivation. Rapamycin or Sirolimus has been approved by the United States Food and Drug Administration (USFDA) and in Europe for clinical therapy as an antiproliferative and immunosuppressant, for renal cell carcinoma and restenosis. First isolated from *Streptomyces hygroscopicus* from soil samples of Easter Island (Rapa Nui), it is a naturally occurring macrolide triene antibiotic with broad activity against yeast and fungi. Sirolimus bound to its intracellular receptor FKBP12, is a highly specific, allosteric inhibitor of mTORC1 in a ternary complex. Although widely used to study mTORC1 signalling for decades, the precise molecular actions of rapamycin are not completely understood. A Rapamycin/FKBP12 complex, by binding the FRB domain of mTORC1, precludes its association with Raptor, thus uncoupling it from its key substrates. While mTORC1 is allosterically inhibited by Rapamycin, mTORC2 is resistant to acute Rapamycin treatments and only long term treatments affect mTORC2 activity by preventing *de novo* assembly of complex 2 (Laplante & Sabatini, 2012; Loewith et al., 2002; Zoncu et al., 2011). Although initially promising, Rapamycin's therapeutic and clinical uses as an anti-neoplastic, encountered road-blocks. S6 kinase's feedback attenuation of insulin/IGF-1 signalling is abolished by Rapamycin treatment, resulting in severe upregulation of PI3K-Akt proliferation and survival signals. S6K inhibition also activates Mek-Erk cascades, and platelet-derived growth factor transcription, which together counteract Rapamycin action and dampen its effects, making it more cytostatic than cytotoxic, a limitation for its use in cancer therapy. Systemically, besides its strong immunosuppressive effects, Rapamycin also has poor solubility and pharmacokinetic properties, which led to the rapid development of several analogues collectively termed "Rapalogs" with lower effective doses and improved pharmacokinetic properties and many are currently undergoing clinical trials (Zaytseva, Valentino, Gulhati, & Evers, 2012). Catalytic or ATP-competitive mTOR kinase inhibitors, Torin1 and Torin2 as examples/prototypes, have also gained considerable importance as they inhibit all known mTORC1 and mTORC2 actions, namely mTORC1 phosphorylation of

4EBP1 and mTORC2 phosphorylation of Akt downstream of PI3K, which is an important survival signal, undesirable in cancer therapy (Liu et al., 2012; Thoreen et al., 2009). Owing to the complex circuitry of the pathway and several other patient factors/pharmacological properties the full-fledged effects mediated by novel standalone inhibitors (mTOR) or dual inhibitors (PI3K and mTOR) are yet to be understood. At this stage, it may be too early to draw a conclusion as to the utility of mTOR inhibition in cancer therapy, although several promising candidates have yielded encouraging pre-clinical outcome in animal studies (Ballou & Lin, 2008; Watanabe, Wei, & Huang, 2011).

1.6. Mouse models

Early observations (1950s) providing valuable insights into tuberous sclerosis came from the naturally occurring Eker rat strain, harbouring a germline retrotransposon insertional-inactivation of TSC2 (exon30). Inherited as an autosomal dominant trait, homozygotes are early embryonic lethal and heterozygote animals develop bilateral solid/cystic renal and pituitary adenomas, uterine leiomyomas, splenic haemangiomas and sub-ependymal nodules (SENs). The discovery and mapping of TSC1 and TSC2 as genes associated with tuberous sclerosis rapidly led to the generation of murine models targeting TSC1 and TSC2, which shared clinical features of TSC patients (Table 2 summarises existing rodent models of TSC). Their phenotypic similarity and studies from transgenic expression of TSC2 in Eker rats confirmed their role as tumour suppressors regulating mTORC1. Mid-gestational (E10.5– E12.5) lethality is seen in homozygotes due growth failure, anaemia, hepatic hypoplasia and cardiac hypertrophy. Although inter-strain phenotypic variability has been observed, multiple renal cystadenomas develop and renal cell carcinoma, benign hepatic haemangiomas and non-metastasizing haemangiosarcomas of the limbs and tail, lung adenomas with poor growth potential manifest in these mice. TSC2 heterozygotes display a stronger phenotype compared to TSC1 heterozygotes in general (K. Inoki & Guan, 2009; Napolioni & Curatolo, 2008; *Tuberous Sclerosis Complex Genes Clinical Features and Therapeutics*, 2010).

Table 2: Available rodent models of TSC1 and TSC2 alleles

Species	Gene	Allele name	Targeted region	Major features of heterozygote animals
Rat	Tsc2	Eker	IAP element insertion into codon 1272	Cystadenomas-carcinomas of kidney, Splenic haemangiomas, Uterine leiomyomas, Pituitary adenomas, Sub-ependymal and subcortical hamartomas
Mouse	Tsc2	-, Kwiatkowski	Neomycin cassette insertion into exon 2	Cystadenomas of kidney, Liver haemangiomas, Extremity angiosarcomas
Mouse	Tsc2	-, Hino	Exon 2 Neo^R-cassette insertion, Exons 2-5 deletion	Cystadenomas of kidney, Liver haemangiomas
Mouse	Tsc1	-, Hino	Exons 6-8 Neo^R-cassette insertion and deletion	Cystadenomas of kidney, Liver haemangiomas
Mouse	Tsc1	-, Kwiatkowski	Exons 17 and 18 deletion	Cystadenomas of kidney
Mouse	Tsc1	-, Cheadle	Exons 6-8 deletion with insertion of Neo^R-cassette	Cystadenomas of kidney, Liver haemangiomas, Reduced survival of $Tsc1^{+/-}$ when in C57BL/6 strain, $Tsc1^{+/-}$ kidney cancer in BALB/c strain
Mouse	Tsc2	Neo, Gambello	Neo^R-cassette insertion into exon 1	Hypomorphic allele: renal cysts only at 20 months of age , and $Tsc2^{neo/neo}$ embryos survive to E17 in some cases
Mouse	Tsc2	Del3, Kwiatkowski	Exon 3 deletion	Hypomorphic allele: reduced severity of renal tumors, 1-2 day longer survival of $Tsc2^{del3/del3}$ embryos
Mouse	Tsc2	KO, Gambello	Exons 2-4 deletion	Cystadenomas of kidney little published data
Mouse	Tsc2	-, Kobayashi	Exons 2-4 Deletion	little published data

Adopted from Tuberous Sclerosis Complex: Genes, Clinical Features and Therapeutics – edited by David J Kwiatkowski, Vicky Holets Whittemore, and Elizabeth A. Thiele, pp. 135, Wiley-Blackwell, 2010.

1.7. mTORC1 in stress response

The need for cells to assimilate multiple signals promoting growth, cell cycle progression or under dire circumstances, initiate death programmes like apoptosis also necessitates integration of signal responses into a major hub of signalling pathways. Although best studied for its control on protein synthesis (Thoreen et al., 2012), mTOR also regulates transcription in response to a variety of conditions, cell cycle progression, actin organization and cytoskeletal dynamics, autophagy etc. (Fingar and Blenis, 2004). Given the plethora of processes mTOR controls, it is no surprise that a vast array of extrinsic and intrinsic factors

feed into and directly impact the pathway. Over the last decade, stimuli of various natures impinging on mTOR have been described (Sarbassov, Ali, & Sabatini, 2005). Adverse nutrient conditions and energetic stress rapidly decrease protein synthesis predominantly through AMPK-dependent activation of TSC1/2 in order to conserve cellular energy levels (Ken. Inoki et al., 2003). A direct outcome is the initiation of autophagic programmes, a major means of catabolic energy production to overcome insufficiency (Høyer-Hansen & Jäättelä, 2007; J. Kim et al., 2011). Accordingly, TSC-/- cells are hypersensitive to glucose deprivation and undergo p53-dependent apoptosis (R. G. Jones et al., 2005; Lee et al., 2007). mTORC1 inhibition confers protection against death during glucose deprivation by decreasing AMPK activation and sustaining ATP levels. This effect was attributed rather to decreased anabolic processes reducing energy consumption, and not to increased autophagy (R. T. Abraham & Eng, 2010). Glutamine anaplerosis and metabolism via TCA cycle deserves a special mention, as several glucose-addicted cancer cells including TSC-/- cells rely almost solely on glutamine for survival under nutrient shortage (Li et al., 2015). mTORC1 inhibition during energetic stress therefore implies a prudent attempt to equate/appropriate metabolic demand with supply (Choo et al., 2010). In addition, decreased Insulin IGF-1/mTOR signalling activity is associated with increased resistance to some types of stress, suggesting that this pathway plays an important role in the adaption to different stress conditions (Scott et al., 2002; Holzenberger et al., 2003; Broughton et al., 2005; Teleman et al., 2005; Tettweiler et al., 2005; Powers et al., 2006).

Faithful transmission of genetic information on the face of constant threat by sources and agents of DNA damage requires robust damage response and repair mechanisms. The tumour suppressor p53 serves to arrest cell cycle, induce repair processes and in the event of irreparable damage, initiate apoptosis to eliminate compromised cells. p53 responds to myriad stress stimuli. Transient activation of mTORC1–S6K axis by p38alpha MAPK results in tight physical interaction (and MDM2 phosphorylation) between S6K and MDM2, thereby inhibiting p53 ubiquitination, promoting its induction and stabilisation to carry out effector functions such as DNA damage-induced cell cycle arrest, apoptosis and/or senescence. Constitutive S6K activity in TSC-/- cells appears to facilitate p53 induction. Transcriptional p53 target genes Sestrin1 (Sesn1) and Sestrin2 (Sesn2) activate AMPK, thus stimulating the TSC2 GAP activity to inhibit mTORC1 and cell growth. Hence p53 stabilisation in TSC1-/- cells has been shown, at least in part, to be responsible for glucose-deprivation and DNA

damage-induced cell death (Lee et al., 2007). However, the hypersensitivity to metabolic and genotoxic stress in the absence of p53 (Choo et al., 2010) suggests that constitutive mTORC1 activity predisposes TSC-/- cells independent of p53 to stress-induced death. Indeed, only recently, energetic insufficiency due to a demand–supply gap and a metabolic collapse was attributed to their heightened stress sensitivity (Choo et al., 2010).

1.8. The mammalian cell cycle and the role of mTORC1 in cell cycle control

The cell cycle entails an ordered progression of four stages: G1, S (DNA synthesis phase), G2, and M (cell division – mitosis). The fidelity of the process, i.e., faithful replication of DNA (G1–S transition) and its uniform distribution into daughter cells (G2–M transition), is ensured by stringently controlled, sophisticated mechanisms. Quiescence, or G_0 phase, is a non-dividing dormant state, a state of 'exit' from the cell cycle. The entire process of transition from the quiescence to proliferation is carefully orchestrated by precisely timed activities of Cyclin–cyclin-dependent kinase (CDK) complexes. Upon mitogenic stimulation, cyclin D–CDK4/CDK6 complex phosphorylates retinoblastoma (Rb) protein, thereby activating the transcription factor E2F. This turns on genes for cyclinE and cyclinA, whose products in a complex with CDK2 promote entry and progression through S phase. The G1 CDKs are negatively regulated by small inhibitory molecules, including p21 and p27 among several others (Berridge, 2012).

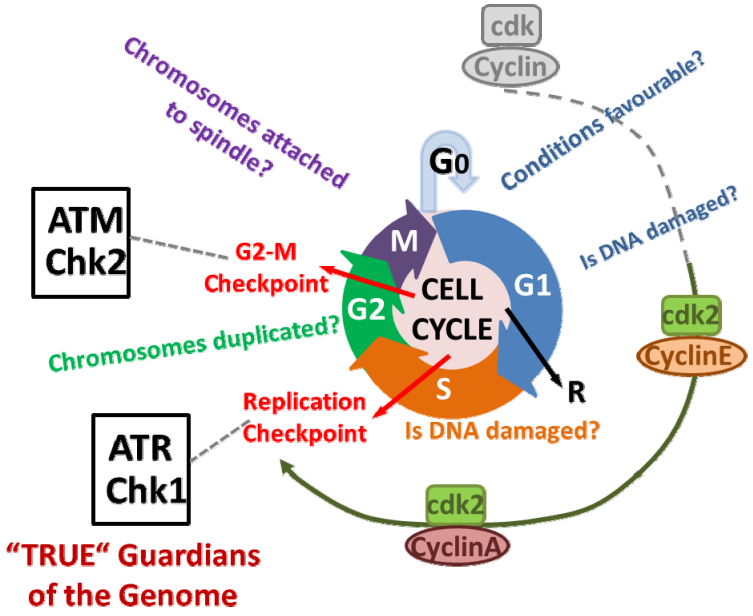


Figure 4: Simplified scheme of the mammalian cell cycle
Entry from quiescence into the cell cycle is guided by orchestrated activity of cyclin-Cdk complexes in an ordered progression of phases aimed at 1. Faithful duplication of genetic material and 2. Equal distribution of the genome into two daughter cells. The fidelity of the entire process is ensured by 'checkpoints' at transitions from either gap-phases G1 or G2 into S and M phases respectively. Additional checkpoints reassure complete DNA replication (Intra-S) and accurate chromosome segregation during mitosis (Mitotic).

mTORC1 gained attention as a cell cycle regulator after its discovery as Rapamycin's cellular target (Eric J. Brown et al., 1994) and following the observation that rapamycin functions as an immunosuppressant by blocking T-cell proliferation in the early 1990s. This ability of Rapamycin to block G1–S transition was extended to several tumour cell types in due course (Robert T. Abraham & Wiederrecht, 1996). In addition to identifying the obligate requirement of S6K activity for G_1–S transition (Ekim et al., 2011; Fingar & Blenis, 2004), much work has focused on the control of expression of Cyclins D, E, A, and the Cdk inhibitor p27 by mTORC1 (Hengstschläger & Rosner, 2003). p27 in particular is of major importance, since it is the chief gatekeeper of the quiescent status in mammalian cells. The high p27 levels in quiescent cells decreases following mitogenic stimulation. Rapamycin treatment can thus lead to the accumulation of inactive cyclin E/Cdk2/p27Kip1 complexes (T. Soucek et al., 1998). Because S-phase entry requires active cyclin E/Cdk2 complexes, binding of p27Kip1 blocks cell-cycle progression. Although far less is known, recent work suggests mTORC1 may well play a role in controlling mitotic entry of cells indirectly, by modulating Cdk1 activity, thus influencing the mitosis promoting function of Cdk1/Cyclin B

complex. mTORC1 signalling controls both G1–S transition and mitotic entry in eukaryotes (Wang & Proud, 2009).

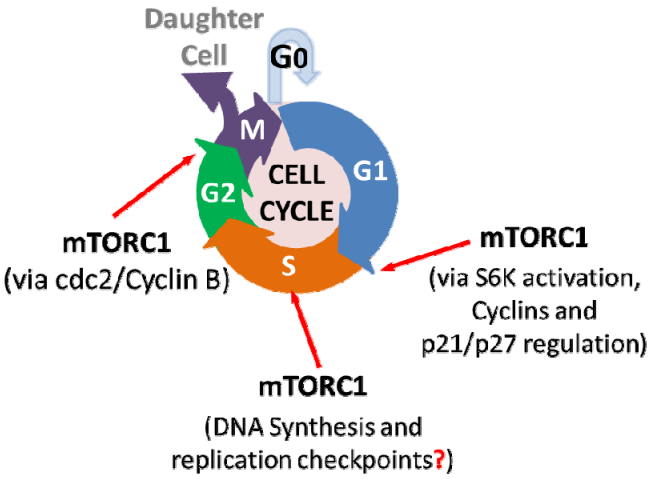


Figure 5: Cell cycle control by mTORC1.
Studies employing mTORC1 inhibitor rapamycin revealed that S6K activation is obligatory for G1-S transition. Recent data also indicate mTORC1's involvement in control of mitotic entry of cells. Besides regulating expression of G1 and S phase cyclins, mTORC1 bears strong homology with other members of the PIKK family e.g., ATR and whether mTORC1 plays a role in S-phase is unknown.

DNA replication is the most vulnerable cellular process that can lead to genomic instability (Osborn, Elledge, & Zou, 2002). RS, simply defined as slowing or stalling of replication forks, derives from a variety of sources including 1. Nicks and gaps (ssDNA), 2. Unrepaired lesions (e.g., aldehyde/protein –DNA adducts), 3. Ribonucleotide misincorporation, 4. Intrinsic DNA structures (e.g., trinucleotide repeats and replisome slippage), 5. G-quadruplexes in GC-rich regions, 6. Replication – Transcription collision and lastly 7. Oncogene activation, has been recognised as an early hallmark in tumorigenesis (Bartkova et al., 2006; Gaillard, Garcia-Muse, & Aguilera, 2015; Lambert & Carr, 2013). H-ras, myc and cyclinE overexpression or constitutive activity are accepted causes of RS and other oncogenes, mos, cdc25A and E2F1 for instance, are novel inclusions in the list. Unchecked RS contributes to genomic instability in cancers driven by these oncogenes. Although how exactly oncogenes cause RS is still a mystery, investigators have hypothesised that oncogenes somehow control the loading of replication factors on potential origins, since oncogene-induced RS almost certainly is an outcome of inappropriate use (over or under-firing) of replication origins (Gaillard et al., 2015; R. M. Jones et al., 2013; Lecona & Fernandez-Capetillo, 2014). Since mTORC1 is known to control not only the expression of G1/S cyclins but also G1-S transition, and since the

prototypic RS oncogene ras leads to mTORC1 activation downstream, it will be interesting to understand whether TSC-/- cells experience replication stress.

Down-regulation or complete loss of TSC2 (Tuberin) expression has been shown to spark cell cycle entry of G_0-arrested cells, prevent quiescence upon serum withdrawal and further drive G1–S transition (Hengstschläger et al., 2001; Thomas. Soucek, Pusch, Wienecke, DeClue, & Hengstschläger, 1997). Moreover, endogenous p27 was shown to be unstable and ectopically overexpressed p27 mislocalised and incapable of cdk inhibition in TSC2-/- cells. This is evidenced by an induction of Cdk2 activity in TSC2-/- cells (T. Soucek et al., 1998). Consistently, analysis of patient material, both TSC1 and TSC2 cases alike, showed a prolonged S phase (Wataya-Kaneda et al., 2001). Together, high mTORC1 activity by way of protein abundance and increased cell mass, along with high G1 Cdk activity appear to shorten the length of G1 phase and drive premature S phase entry in TSC-/- cells, although the exact duration of S phase in TSC-/- cells and whether they experience replication issues rendering them hypersensitive to stress is still not clear.

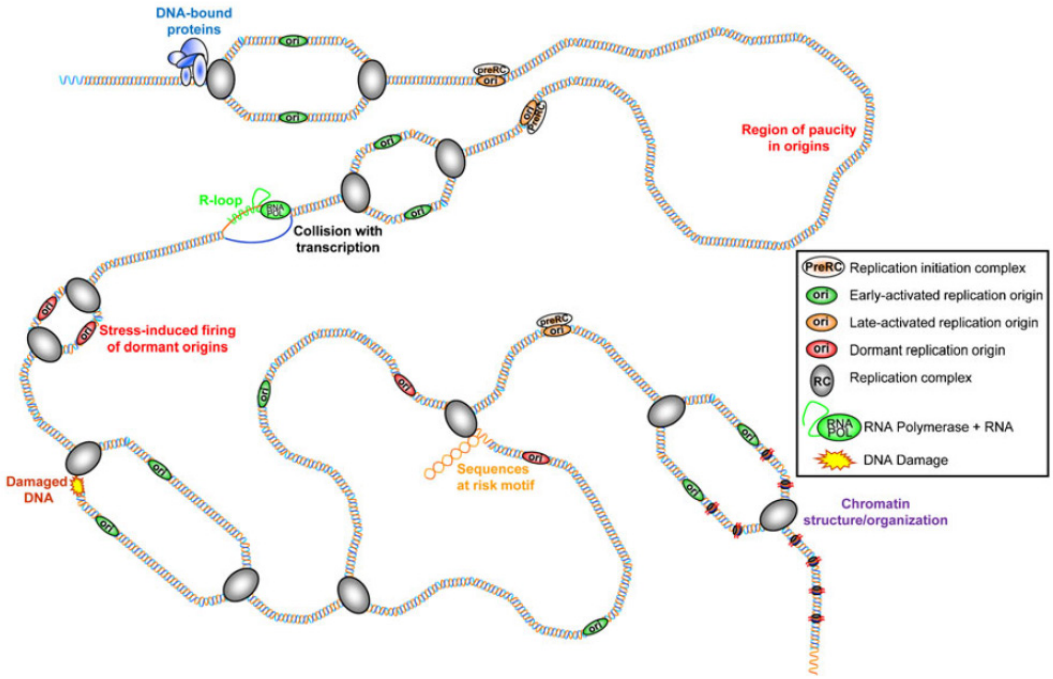


Figure 6: Sources of local and global replication stress.
RS, defined as slowing or stalling of replication forks, derives from a variety of sources including nicks and gaps (ssDNA), unrepaired lesions (e.g., aldehyde/protein –DNA adducts), ribonucleotide misincorporation, intrinsic DNA structures (e.g., trinucleotide repeats and replisome slippage), G-quadruplexes in GC-rich regions, R-loops and replication – Transcription collision, and lastly oncogene activation. Oncogene-induced replication stress is an early hallmark in tumorigenesis, and is an outcome of inappropriate replication origin usage. Source: Reproduced with permission from Prof. Sarah Lambert, Institut Curie, centre de recherche, CNRS, Orsay, France.

1.9. Open questions

mTOR has gained protracted attention over three decades in the context of a multitude of cellular and physiological functions, but studies pertaining to its role in metabolic/energy stress response far superseded those on genotoxic stress response until recently, after prolific interest in mTOR as a major cell cycle regulator. While energetic stress has devastating consequences for TSC-/- cell survival, due to their endogenous energy shortfall and not the apparently poor survival signal due to persistent S6K-IRS1-Akt feedback inhibition, these cells have also been shown to be hypersensitive to mild genotoxic stress under standard (energy-replete) growth conditions. This is of particular interest in view of the therapeutic potential of selectively killing TSC tumours, while leaving normal cells unperturbed. Acute genotoxic stress is known to invoke a robust p53 response in TSC-/- cells, although the fact that this remains futile in terms of initiation of cell cycle arrest and/or repair processes is intriguing and several questions arise, owing to the lacuna in our current understanding of cell cycle regulatory properties of mTORC1.

What maintains TSC-/- tumours in a benign state? What is the nature and outcome of constitutive mTORC1 activity on cell cycle progression and regulation in TSC-/- cells? Do TSC-/- cells experience oncogene-driven replication stress? If yes, what is the link between anomalous cell cycle regulation, DNA damage recognition and repair processes; and how such alterations predispose TSC-/- cells to sub-lethal genotoxic stress-induced death? Why do TSC-/- cells fail to adapt to mild stress on a backdrop of a vigorous growth-promoting pathway? Finally, how does the energetic well-being or ill-being of the cell influence the outcome of TSC-/- cells subjected to genotoxic stress?

2. HYPOTHESIS AND AIMS

2.1. Hypothesis

The mTORC1 pathway, with its core components – the Ser/Thr kinase mTOR and its upstream negative regulator, the tumour suppressor complex TSC1/TSC2, is the principal intracellular integrator of several extrinsic inputs including nutrient and energy sensing, insulin signalling, and stress, controlling cell mass and growth (Laplante & Sabatini, 2012).

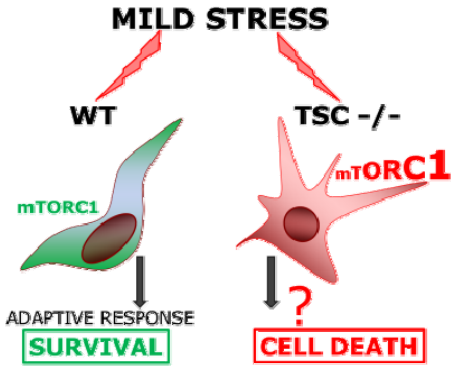


Figure 7: TSC-/- cells forfeit adaptation to mild stress.
TSC-/- cells succumb to mild-stress. Exactly how a strong growth-promoting pathway sensitises cells to such low-dose stress is the subject of investigation.

Aberrant high mTORC1 activity is causally linked to several major human pathophysiologies including metabolic syndromes like insulin resistance, diabetes and cancer. Paradoxically, syndromes with mTORC1 hyperactivity exhibiting poor growth and proliferation potential also exist. Loss of either component of the tumour suppressor complex (TSC1 or TSC2) gives rise to a heterogeneous class of neoplasias called hamartomas. Despite resulting in high mTOR activity, loss of TSC function leads to the formation of highly apoptotic neoplasias without malignancy. A similar clinical picture applies to the Peutz-Jeghers neoplasia driven by AMPK loss, a second major upstream regulator of mTORC1 that integrates energy scarcity signals. These observations suggest that mTORC1 functions as a "tumour promoter" facilitating aberrant growth but is unable to drive oncogenic transformation. Along with other lines of evidence this observation portrays aberrantly high mTORC1 activity as a critical sensitizer of transformed cells to DNA damage/stress induced cell-cycle arrest and/or apoptosis, in marked opposition to its well established growth-promoting role in cases of moderate mTOR activation. This represents a classical hormetic response (Calabrese, 2013; Calabrese & Baldwin, 2001; Gems & Partridge, 2008; Löffler, Grün, Böhmer, & Rubio, 2008)

and as such points to an adaptive response of cells to the intensity of signal flux via the Rheb/mTOR pathway. This hormetic behaviour would explain the benign nature of Tuberous sclerosis and could also play a role in metabolic syndromes like Diabetes type II, which are characterized by a low response to high insulin start-up signals. Previous studies have identified translational activation of pro-apoptotic and anti-proliferative factors as a cause of such sensitivity of TSC-/- cells to sub-lethal stress (Freilinger et al., 2006; Lee et al., 2007). More recently, energetic insufficiency and a metabolic collapse, due to increased demand and lack of resource-sensing mechanisms (loss of TSC function) respectively, have been shown to contribute to the underlying mechanisms of stress-sensitivity (Choo et al., 2010). Anomalous control of cell cycle progression has also been implicated (T. Soucek et al., 1998).

2.2. Aims

Together with evidence from previous reports, we hypothesised that exorbitantly high mTORC1 activity, as in TSC-/- cells, compromises the ability of cells to cope with and adapt to sub-lethal, hormetic levels of genotoxic stress. The specific aims of the current project are:

1. Characterise the magnitude of effect and the nature of insults in response to which mTORC1 activity levels dictate cellular fate (death outcome).
2. Decipher mechanisms by which mTORC1 controls cell cycle progression and crosstalks with DNA damage response machinery to fulfil its sensitising/predisposing function in TSC-/- cells. In other words, mechanisms contributing to the loss of adaptive capacity in TSC1-/- MEF cells.

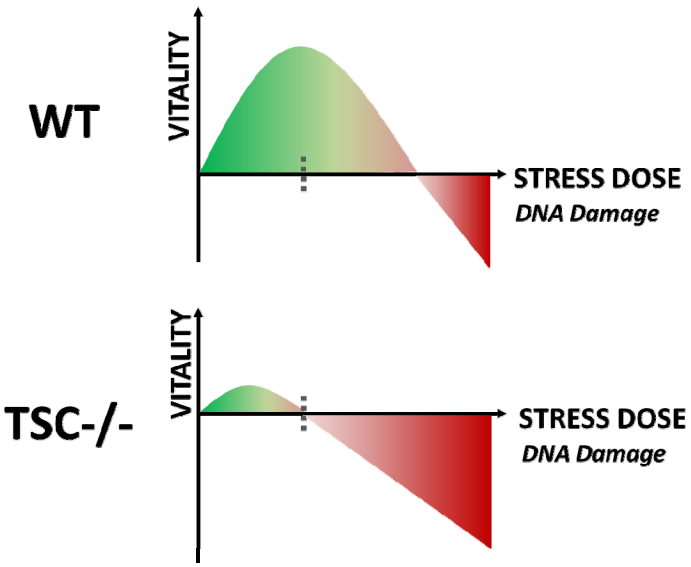


Figure 8: Skewed hormetic response in TSC-/- cells owing to the derangement of the mTORC1 pathway

The term "hormesis" encompassing various adaptive phenomena of cellular systems evident from non-linear responses (for e.g., viability) to a linear increase in external stress doses (for e.g., glucose starvation or drugs) is under intense investigation in efforts to understand cellular stress-response. We hypothesise that superlative mTORC1 activity levels skew the hormetic range of TSC-/- cells to their detriment, as though they have 'lost' their adaptive capacity to otherwise harmless stress doses of diverse nature.

3. Materials and Methods

3.1. Materials

Table 3: Cell Culture Media and Supplements and transfection reagents

Name	Vendor	Catalogue No.
Accutase	Sigma-Aldrich Co. LLC.	A6964
DMEM	Sigma-Aldrich Co. LLC.	D6429
DMEM no glucose, no phenol red, no glutamine	Gibco® by Life technologies	A14430
DMEM High Glucose	PAA Laboratories GmbH	E15-843
DMEM Low Glucose	Biowest LLC	A0330
DPBS	Gibco® by Life technologies	14040-117
EmbryoMax® 100x Nucleosides	Merck Millipore Corporation	ES-008-D
FCS	Biowest LLC	S1810-050
GlutaMax	Gibco®by Life technologies	35050-038
Lipofectamine RNAiMAX	Invitrogen /Life Technologies	13778-150
MEM Amino acids	PAA Laboratories GmbH	M11-002
MEM Vitamins	Gibco® invitrogen™	11120-037
OptiMEM medium	Invitrogen, life technologies,	31985-070
Sodium pyruvate	PAA Laboratories GmbH	S11-003
Trypsin-EDTA	Gibco®by Life technologies	25300-045

Table 4: General Laboratory chemicals and reagents

Name	Vendor	Catalogue No.
(+)-Sodium-L-ascorbate	Sigma-Aldrich Chemie GmbH	A4034
2-Deoxy-D-Glucose	Fluka Biochemika	31066
Acetic acid	Carl Roth GmbH & Co. KG	3738.5
Rotiphorese® Gel 30 30 % Acrylamide/Bis-acrylamide (37.5:1)	Carl Roth GmbH & Co. KG	3029.1
Ammonium persulphate (APS)	SERVA Electrophoresis GmbH	13375
bisBenzimide H 33342 trihydrochloride	Sigma-Aldrich Chemie GmbH	B2261
Bromophenol blue	Carl Roth GmbH & Co. KG	A512.1
Bovine serum albumin (BSA)	PAA Laboratories GmbH	K41-001
Calcium chloride ($CaCl_2$)	AppliChem GmbH	A4689,0250
Collagen	Sigma-Aldrich Chemie GmbH	C7661
Coomassie Brilliant Blue G-250	Carl Roth GmbH & Co. KG Fluka Biochemika	9598.1 27815
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH	D2650
Dithiothreitol (DTT)	AppliChem GmbH	A2948,0010
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG	8043.2
Ethylene glycol tetraacetic acid (EGTA)	AppliChem GmbH	A0878,0025
Ethanol	Carl Roth GmbH & Co. KG	K928
Glucose	Merck KGaA Gibco BRL	8337 39002-019
Glycerol	Carl Roth GmbH & Co. KG	3783.2
Glycine PUFFERAN®	Carl Roth GmbH & Co. KG	3908.3
HEPES	AppliChem GmbH	A1069,0500
Isopropanol	Carl Roth GmbH & Co. KG	6752.4
Leupeptin-hemisulphate	AppliChem GmbH	A2183, 0025
Magnesium chloride ($MgCl_2$)	Carl Roth GmbH & Co. KG	HN03.1

2-Mercaptoethanol	Fluka Biochemika	63690
Menzel-Gläser SUPERFROST	Thermo Fisher Scientific Inc.	AG00008032E
Methanol	Carl Roth GmbH & Co. KG	3880.2
Microycystin-LR	Enzo Life Sciences GmbH	
Neomycin	Sigma-Aldrich Chemie GmbH	A-1720
Nonylphenylpolyethylenglycol (NP)-40	Merck KGaA	492016
RNase A from bovine pancreas	Roche Diagnostics GmbH	70297721
Okadaic Acid, Sodium Salt	EMD Chemicals, Inc.	#459620
Paraformaldehyde	Sigma-Aldrich Chemie GmbH	158127
Pefabloc SC®(AEBSF hydrochloride)	AppliChem GmbH	A1421,0500
Pepstatin A	AppliChem GmbH	A2205,0025
Phenylmethylsulfonylfluoride (PMSF)	Sigma-Aldrich Chemie GmbH	P7626
Polyethylenimine (PEI) branched	Sigma-Aldrich Chemie GmbH	40,872,7
Potassium acetate	Carl Roth GmbH & Co. KG	4986.1
Potassium chloride (KCl)	Carl Roth GmbH & Co. KG	6781.1
Propidium Iodide	EMD Chemicals, Inc.	537059
ProSieve™ 50 Gel Solution	Lonza	50618
Sodium-β-glycerophosphate	SERVA Electrophoresis GmbH	
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG	3957.2
Sodium Deoxy cholate	Sigma-Aldrich Chemie GmbH	D6750
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Chemie GmbH	A3942,1000
Sodium hydroxide (NaOH)	Sigma-Aldrich Chemie GmbH	9356.1
Sodium orthovanadate	Sigma-Aldrich Chemie GmbH	S6508
Spectra™ Multicolor Broad Range Protein Ladder	Thermo Fisher Scientific Inc.	26634
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Serva Electrophoresis GmbH	35930
Thiazolyl Blue Tetrazolium Bromide	Sigma-Aldrich Chemie GmbH	M5655
Tris ultrapure	AppliChem GmbH	A1086,5000
Triton® X-100	Carl Roth GmbH & Co. KG	6683.1
Tween® 20	SERVA Electrophoresis GmbH	3747.0

Table 5: Kits

Name	Vendor	Catalogue No.
Annexin V-FITC Apoptosis Detection Kit I	BD Biosciences Pharmingen	556547
CFSE labelling kit	Cayman Chemical Co.	10009853
Click-iT® Plus EdU Alexa Fluor® 647 Flow Cytometry Assay Kit	Molecular Probes™	C10634
Micro BCA™ Protein Assay Kit	Thermo Scientific TM	23235

Table 6: Lysis Buffers and Solutions

Cell lysis reagents	Composition
Standard lysis buffer	50 mM HEPES pH 7.5 150 mM NaCl 5 mM MgCl2 1 mM EDTA 1% NP-40
RIPA lysis buffer for nuclear extraction	50mM TRIS-HCl pH 8.0 150mM NaCl 5mM $MgCl_2$ 1% Nonidet P-40 0.5% Deoxycholate 0.1% SDS
Protease and phosphatase inhibitor cocktail	**freshly supplemented from frozen stocks** <u>Protease inhibitors</u> 42 mM Pefabloc 2 µM Leupeptin 100 µM PMSF 1.5 µM Pepstatin A <u>phosphatases inhibitors</u> 100 µM Sodium Vanadate 3.4 nM Microcystin 1 µM β-Glycerophosphate 1µM Okadaic Acid
Spreading buffer for DNA fibre assay	200mM Tris-Hcl, pH- 7.4 50mM EDTA 0.5% SDS

Table 7: Chemicals and agents, mTOR inhibitors, base analogues and siRNA

Name	Vendor	Catalogue #	Stock Solution	Storage	Working conc.
CIdU	SIGMA	C6891	2,5mM	-20°C	0,025nM
Doxorubicin hydrochloride (Adriamycin)	SIGMA	D1515	5µg/mL	-20°C, light protected	0.5µg/mL
Hydroxyurea (HU)	SIGMA	H8627	1M, freshly prepared	-	2mM
IdU	SIGMA	I7125	2,5nM	-20°C	0,25mM
Irinotecan	SIGMA	I1406	100µM	-20°C 3-6 months	100nM
Methyl Methane Sulphonate (MMS)	Sigma-Aldrich GmbH	129925	25mg/ml	-20°C, short term	25µg/ml
mTOR inhibitor XIII [ETP-46464] (used as ATR inhibitor)	EMD Chemicals Inc.	500508	50µM	-20°C	25-50nM
Nocodazole	SIGMA	M1404	100µg/ml	-20°C	100ng/ml
Rapamycin	Calbiochem	553210	20µM	-20°C, up to 3 months	20nM
Ro3306	SIGMA	SML0569	10µM	-20°C	10nM
Torin1	Tocris	4247	10µM	-20°C 3-6 months	2-10nM
Thymidine	SIGMA	T1895	100mM	-20°C	2mM
TSC2siRNA (mouse)	Dharmacon smartpool	L-047050-00-0005	20µM	-20°C	-

Table 8: Antibodies, Primary antibodies and conjugates

Antibody	Vendor	Catalogue No.	Application	Dilution
4E-BP1 Ab	Cell Signaling Technology, Inc.	#9452	Western blotting	1:1000
AMPK	Cell Signaling Technology, Inc.	2532	Western blotting	1:1000
Anti-CHK2, clone 7	Merck Millipore Corporation	05-649	Western blotting	1:1000
Anti-phospho-Histone H2A.X (Ser 139) clone JBW301	Merck Millipore Corporation	05-636	Western blotting	1:1000
Anti-phospho_Histone H2A.X (Ser 139) clone JBW301 FITC conjugate	Merck Millipore Corporation	FCMAB 16-202A	Flow cytometry	1:800
Anti-phospho H3 (Ser10), clone 3H10 Alexa Fluor® 488 conjugated	Merck Millipore Corporation	FCMAB104A4	Flow cytometry	1:50
ATM mAb (2C1)	Novus Biologicals	NB100-309	Western blotting	1:1000
β–Actin mAb	Sigma	A5441	Western blotting	1:1000
ATR (N-19) Goat polyclonat IgG	Santa Cruz Biotechnology, Inc.	sc-1887	Western blotting	1:1000
Caspase 3-Rabbit Ab	Cell Signaling Technology, Inc.	#9662S	Western blotting	1:1000
Cdc 45 (3G10) mAb	In house	-	Western blotting	1:1000
Cdk2 (M2) Rabbit polyclonal IgG	Santa Cruz Biotechnology, Inc.	sc-163	Western blotting	1:1000
Chk1 (2G1D5) Mouse mAb	Cell Signaling Technology, Inc.	#2360S	Western blotting	1:1000
Cleaved Caspase-3 (Asp 175) 5A1 Rabbit monoclonal Ab	Cell Signaling Technology, Inc.	#9664	Western blotting	1:1000
HA-Tag (6E2) (HRP Conjugate) Mouse mAb	Cell Signaling Technology, Inc.	#2999S	Western blotting	1:1000
MDM2 (HDM2-323)	Santa Cruz Biotechnology, Inc.	sc-56154	Western blotting	1:1000
Monoclonal mouse anti-BrdU (Clone B44)	Becton Dickinson Immunocytometry Systems	347580 (7580)	Fibre Assay	1:1500
Monoclonal rat anti-BrdU, Clone BU1/75 (ICR1)	AbD Serotec®	OBT0030	Fibre assay	1:1000
p21 (F-5)	Santa Cruz Biotechnology, Inc.	sc-6246	Western blotting	1:1000
p27 (C-19) Rabbit polyclonal IgG	Santa Cruz Biotechnology, Inc.	sc-528	Western blotting	1:1000
p53 (1C12) Mouse mAb	Cell Signaling Technology, Inc.	#2524S	Western blotting	1:1000
p70 S6 Kinase Rabbit Ab	Cell Signaling Technology, Inc.	#9202S	Western blotting	1:1000

P-Akt (S473)(D9E) XP (R) Rabbit mAb	Cell Signaling Technology, Inc.	#4060S	Western blotting	1:1000
P-AMPK alpha (T172) (40H9) Rabbit mAb	Cell Signaling Technology, Inc.	#2535S	Western blotting	1:1000
PARP Rabbit Ab	Cell Signaling Technology, Inc.	#9542S	Western blotting	1:1000
P-ATM (S1981) (D6H9) Rabbit mAb	Cell Signaling Technology, Inc.	#5883S	Western blotting	1:1000
P-ATR (S248) Rabbit Ab	Cell Signaling Technology, Inc.	#2853S	Western blotting	1:1000
P-Chk2 (T68) Rabbit Ab	Cell Signaling Technology, Inc.	#2661S	Western blotting	1:1000
PERK Rabbit mAB (C33E10)	Cell Signaling Technology, Inc.	#3192S	Western blotting	1:1000
P-Histone H2A.X (S139) Rabbit Ab	Cell Signaling Technology, Inc.	#2577S	Western blotting	1:1000
Phospho 4E-BP1 (Thr70) Ab	Cell Signaling Technology, Inc.	#9455S	Western blotting	1:1000
Phospho-6 Ribosomal Protein (Ser 235/236) Ab	Cell Signaling Technology, Inc.	#2211S	Western blotting	1:1000
Phospho-Tuberin/TSC2 (Ser939)	Cell Signaling Technology, Inc.	#3615S	Western blotting	1:1000
P-p53 (S15) Rabbit Ab	Cell Signaling Technology, Inc.	#9284S	Western blotting	1:1000
P-p70 S6 Kinase (T 389) (1A5) Mouse Ab	Cell Signaling Technology, Inc.	#9206S	Western blotting	1:1000
P-p70 S6 Kinase (T 389) Rabbit Ab	Cell Signaling Technology, Inc.	#9205S	Western blotting	1:1000
P-PERK(T980) Rabbit mAb	Cell Signaling Technology, Inc.	#3179S	Western blotting	1:1000
RB anti-Phospho Chk1 (S317)	BETHYL	A300-163A	Western blotting	1:1000
Rb mAb to Cdk2 (phospho T14) [EP 2234Y]	abcam®	ab68265	Western blotting	1:1000
Rheb Antibody	Cell Signaling Technology, Inc.	#4935S	Western blotting	1:1000
S6 Ribosomal Protein (5G10) Rabbit mAb	Cell Signaling Technology, Inc.	#2217	Western blotting	1:1000
Tuberin/TSC2 (Ser939)	Cell Signaling Technology, Inc.	#3612S	Western blotting	1:1000
Vinculin Antibody	Cell Signaling Technology, Inc.	#4650S	Western blotting	1:1000

Table 9: Antibodies, Secondary conjugates

Secondary antibody	Type	Dilution	Application	Manufacturer
HRP-anti goat	donkey polyclonal	1:10000	Western Blotting	Santa Cruz Biotechnology®
HRP-anti mouse	goat polyclonal	1:10000	Western Blotting	KPL®
HRP-anti rabbit	goat polyclonal	1:10000	Western Blotting	KPL®
HRP-anti sheep	rabbit polyclonal	1:10000	Western Blotting	Santa Cruz Biotechnology®
Goat anti-mouse AlexaFluor488	goat polyclonal	1:500	Fibre Assay	abcam®
Goat anti-rat AlexaFluor555	goat polyclonal	1:500	Fibre Assay	abcam®

3.2. Methods

3.2.1. Cell culture and treatments

Cellular models of Tuberous Sclerosis Complex (TSC), including TSC1-/- (Hamartin null), TSC2-/- p53-/- (Tuberin, p53 double null) and p53-/- (p53 null) mouse embryonic fibroblasts (MEFs) along with their Wild-Type counterparts procured from the Kwiatkowski laboratory, Boston, MA, were maintained in continuous cultures in Dulbecco's Modified Eagle's Media with stable L-glutamine (DMEM; Sigma, #D6429) supplemented with 10 % foetal calf serum under standard conditions (95 % humidity, 5 % CO_2, 37 °C). For various treatments involving growth conditions, cells were cultured in DMEM without D-Glucose and L-Glutamine (Gibco, #A14430) or Dulbecco's PBS (Gibco, #14040-117) supplemented with the indicated nutrient(s), dialysed FCS and carbon energy source. Genotoxic treatments with Hydroxyurea (HU, 2mM) and Adriamycin (Adr, 0.5µg/mL) were for indicated periods as in the relevant results section. A list of supplements used can be found in the materials section.

3.2.2. Protein quantification

Absolute protein concentrations from whole-cell RIPA buffer lysates were quantified using Pierce® BCA Protein Assay Kit following the manufacturer's instructions (Thermo Scientific™; #23225 and #23227). Samples were assayed in duplicates in 96-well microtiter plates and the absorption recorded using a spectrophotometer at 570nm against a known standard BSA concentration range.

3.2.3. SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed using a vertical slab electrophoresis system (Hofer scientific, California, #SE400) and either standard (Rotiphorese, Carl Roth, 3029.1) or 8-10% gradient polyacrylamide gels (Lonza, ProSieve, #50618) depending on the need for resolution. Proteins were separated in SDS running buffer at a constant current of 25-30 mA per gel.

3.2.4. Western blotting and immunodetection of immobilized proteins

Electrotransfer of SDS-PAGE resolved proteins, was carried out with a Trans-Blot Cell Tank system (Bio-Rad™, #49BR37552) according to the manufacturer's instructions. Proteins were transferred on to methanol-water pre-activated 0,45µm or 0,20µm pore-size PVDF

membranes (Millipore, Immobilon-P, #IPVH00010 and ISEQ00010) with 1X TOWBIN blotting buffer for 1.5h with a constant current of 1.5 A for a set of 2 membranes. Membranes were subsequently blocked with 1 % BSA (in 1X TBS-T, PBS + 0.05 % Tween 20®) for 30' at RT. Blots were probed with primary antibody overnight at 4 °C with shaking. Membranes were washed thrice (10 min, 1X TBS-T) and incubated with secondary antibody (1:10,000 in 1 % BSA 1X-TBS-T) for 1h at RT with shaking. Following three further washing steps, membranes were flushed with Western Lightning PLUS-Ecl Enhanced Chemiluminescent Substrate (PerkinElmer; #ORT2655 + ORT2755) and the bioluminescence signal detected with the FuJiFilm LAS-2000 documentation system. Antibodies used are listed in detail in the materials section.

3.2.5. siRNA–mediated acute knockdowns

TSC2 siRNA (Dharmacon, Smart Pool, # L-047050-00-0005) and Tp53 siRNA (Dharmacon, Smart Pool, #L-040642-00-0005) targeting expression in MEF cells were transfected into WT MEFs and TSC1-/- MEFs respectively, using Lipofectamine® RNAiMAX reagent (Invitrogen, Life Technologies #13778) as per the vendors' recommendations. 25nM siRNAs were transfected in cells right after being seeded at a density of 30–50 % confluency depending on experiments by using Lipofectamine RNAiMAX (Invitrogen, #13778-150) according to the manufacturer's protocols. All transfections were performed in 6-well plates 1 day after seeding 3-5 x 10^4 cells per well. Briefly, Lipofectamine RNAimax reagent as well as the siRNA (1.5µL from 20µM stock) was diluted 1:30 (5µL to 150µL) in OptiMEM medium (life technologies, #31985070), mixed and allowed to complex by incubating 5'-10' and gently overlaid on cells dropwise carefully covering all areas. Plates were incubated under standard conditions (95 % humidity, 5 % CO_2, 37 °C) for 48 – 72 h, subjected to treatments and harvested accordingly, for western blotting or flow cytometry.

3.2.6. ATP measurement

Extracts for ATP measurement were prepared as follows. Briefly, at the end of treatments, MEF cells in 6-well plates were lysed in 0.5mL of 96% ethanol, allowed to evaporate (air-dry) or blown-dry, solubilized in 0.5mL Tris-EDTA buffer (100mM Tris-HCl, 2mM EDTA) by freeze-thawing the plate in liquid nitrogen, and the suspension collected with the aid of cell-scraper into 1.5mL Eppendorf tubes. Samples were centrifuged at 14,000rpm for 10' and

the supernatant transferred into fresh Eppendorf tubes. Samples may be stored frozen at -20 °C for up to 2 weeks. Samples were diluted serially in two steps of 1:25 in a final volume of 800µL prior to determining ATP levels by luciferin-luciferase luminometry employing the kit (Biothema ATP SL, Cat# 144-041) as per the manufacturer's instructions. Duplicate treatments were included for total protein estimation by microBCA assay so as to express ATP levels per µg of total cellular protein and for western blotting.

3.2.7. Flow cytometry

3.2.7.1. PI exclusion for cell death

MEF cells, either untreated or treated as indicated, were harvested by Accutase–treatment and pooled with media supernatant to gather detached, dead cells, centrifuged at 700g, washed once in cold wash buffer (PBS with 5 % FCS, 4.5g/L D-glucose, MEM vitamins), resuspended in 300µL of the buffer containing Propidium Iodide (Calbiochem, EMD chemicals, Inc., #537059) to a final concentration of 1.5µg/mL. Samples were analysed on a FACS canto (BD Biosciences) instrument equipped with a 633-nm red laser. The MEF cell population was gated-in with a FSC/SSC dot plot and doublets gated-out based on a DNA dye area/width dot plot. This cell population was further analysed for PI positivity on histogram plots, and percent PI positive cells were accounted as non-viable. *FlowJo* software was used for analysis and quantification.

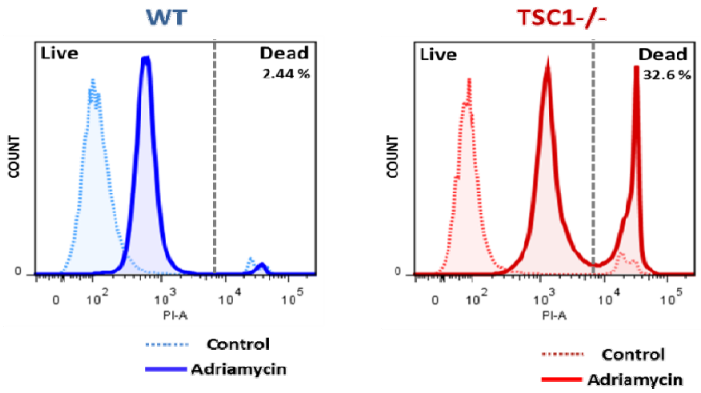


Figure 9: Principle and gating strategy for PI exclusion membrane integrity-based cell death assay. Notice the elevated PI positive cell counts in the TSC1-/- sample treated with genotoxic agent adriamycin (clear red), compared to both WT cells, treated or untreated (blue) as well as TSC1-/- control sample (dotted red). The rightward shift of the 'live' cell peak in treated samples of both cell types is due to background fluorescence caused by membrane-binding of the bright orange coloured drug adriamycin.

3.2.7.2. EdU incorporation ClickIT for cell cycle analysis

Cells were pulsed with the thymidine analogue 5-ethynyl-20-deoxyuridine (EdU) for 20', harvested by Accutase–detachment and stained for DNA synthesis and cell cycle distribution using the Click-iT EdU-AlexaFluor647 Flow Cytometer Assay kit (Molecular Probes, Life Technologies, #C10635), following the manufacturers' protocol. EdU was "clickIT-coupled" to AlexaFluor647 and DNA content was determined by 40,6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, Invitrogen, #D1306) staining. In addition, the cells were stained with either anti-phospho^{Ser10}-histoneH3–AlexaFluor488 antibody conjugate (Millipore, # FCMAB104A4) that specifically labels M-phase cells or anti-γH2Ax–FITC antibody conjugate (Millipore, #16-202A) as a marker of DNA damage. Samples were subjected to multi-colour flow cytometry on a FACS Canto II (BD Biosciences) cytometer equipped with blue (488-nm), red (633-nm) and violet (405-nm) lasers. The MEF cell population was gated-in with a FSC/SSC dot plot and doublets gated-out based on a DNA dye area/width dot plot. This cell population was further analysed for its cell cycle distribution. G1-, S- and G2/M-phase cell populations were defined in a DNA dye/EdU-Alexa Fluor 647 dot plot and G2/M phase cells were further separated in a DNA dye/AlexaFluor 488 dot plot. *FlowJo* software was used for analysis and quantification.

3.2.7.3. Hoechst 33342/PI dual staining

MEF cells, either untreated or treated as indicated, were fed with membrane-permeable live-cell stain Hoechst33342 (bisBenzimide H 33342, SIGMA, #B2261) for 30', harvested by Accutase–treatment and pooled with media supernatant to gather detached, dead cells, centrifuged at 700g, washed once in cold wash buffer (PBS with 5%FCS, 4.5g/L D-glucose, MEM vitamins), resuspended in 300µL of the buffer containing Propidium Iodide to a final concentration of 1.5µg/mL. Samples were analysed on a FACS canto (BD Biosciences) instrument equipped with a 633-nm red laser. The MEF cell population was gated-in using a FSC/SSC dot plot and doublets gated-out based on a DNA dye area/width dot plot. This cell population was further analysed for DNA content i.e., Hoechst positivity (linear scale) vs PI positivity (log scale) on dot plots, so as to correlate cell cycle stage with cell death. *FlowJo* software was used for analysis and quantification.

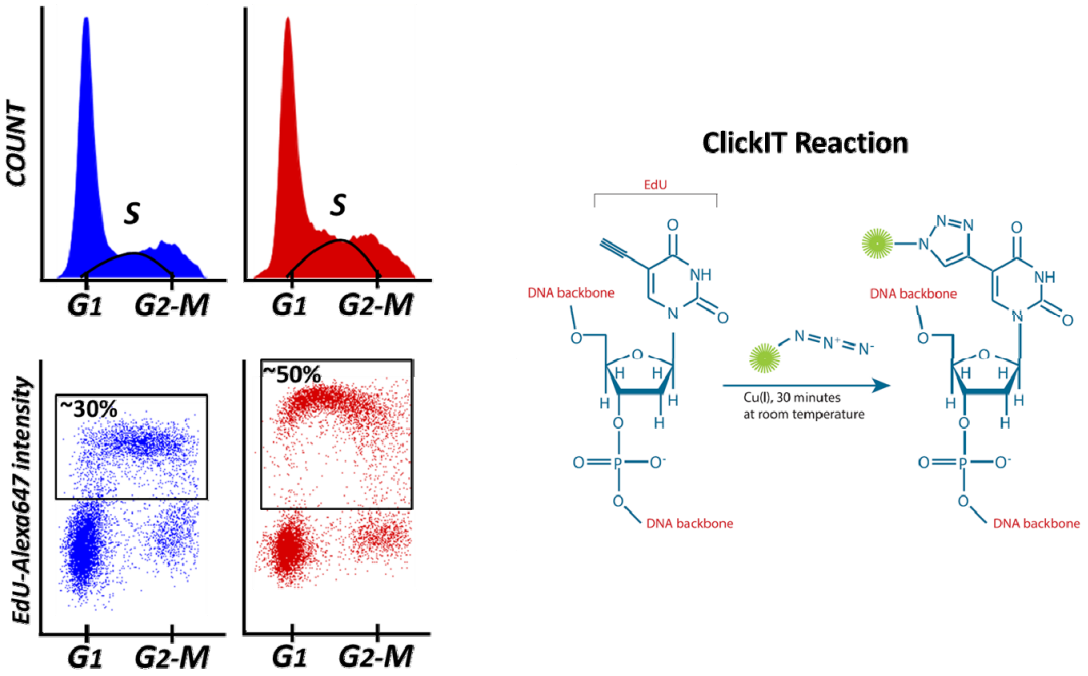


Figure 10: Principle and advantage of the two dimensional multi-colour EdU-incorporation ClickIT Flow cytometry over conventional cell cycle analysis.
Thymidine analogue (nucleotide base) incorporation flow cytometry projects a more realistic picture of the DNA synthesis (S) phase. Unlike other analogues for e.g., BrdU requiring extensive denaturation of DNA and antibody-based detection, the EdU (alkyne group) incorporation method is based on a copper-catalysed chemical "ClickIT" coupling with a reactive azide-fluorophore (usually Alexa dye), making the method rapid and robust. Notice the immediate advantage of precise S-phase quantification compared to the analysis of the conventional cell cycle format. The compatibility of ClickIT reaction with other antibody-based staining opens further avenues for multi-parametric analyses for e.g., phospho-Histone H3 (Ser10) to mark mitotic cells within the G2-M population, and so on.

3.2.7.4. DNA Fibre Spreads and Immunofluorescence

DNA fibres were prepared by on-slide lysis and gravity-spreading as described originally by Jackson and Pombo (Jackson & Pombo, 1998). Exponentially growing MEF cells, untreated and treated in either adherent T_{25} flasks or 60mm dishes were tandem pulse-labelled for 20' in standard growth medium (DMEM + 10 % FCS) with 25 mM CldU and 250 mM IdU respectively, washed once with ice-cold PBS, and collected by scraping. Roughly 1,000 cells in suspension were lysed in a droplet of 7µL spreading buffer (200mM Tris-HCl pH 7.4, 50mM EDTA, 0.5 % SDS) for 2' on one end of grease-free microscopic slides and the chromatin spread by slide-tilting and gravity flow of the droplet over several minutes. Once spread and dry, fibres were fixed for 10 min in 3:1 methanol:acetic acid, the slides air-dried, rehydrated, the DNA denatured with 2.5M HCl for 75 min, washed and incubated in blocking

buffer (PBS, 1 % BSA, 0.1 % Tween-20) for 1 hr. Fibres were labelled with rat anti-BrdU antibody (1 h, 1:1000, ab6326; Abcam), fixed in 4% PFA for 10 min, and sequentially labelled with anti-rat AlexaFluor 555 antibody (2 h, 1:500; Molecular Probes), mouse BrdU antibody (overnight at 4°C, 1:1,500, 347583; BD), and anti-mouse AlexaFluor 488 antibody (2 h, 1:500; Molecular Probes). Slides were mounted, images acquired under oil-immersion (100X) with an Olympus BX61 immunofluorescence microscope and analysed using ImageJ software (http://rsb.info.nih.gov/ij/). CldU and IdU track lengths were measured using ImageJ and appropriate conversions applied for pixels-to-micrometres-to-kilobases as described earlier by Jackson and Pombo. Over 200 replication forks were analysed. Wherever indicated, counts of Origins, Terminating and elongating structures were determined using the cell counter plug-in for ImageJ. Fibre tracks were categorised; red-green (ongoing replication), red (stalled or terminated forks), green (2nd pulse origin) and red-green-green-red (1st pulse origin).

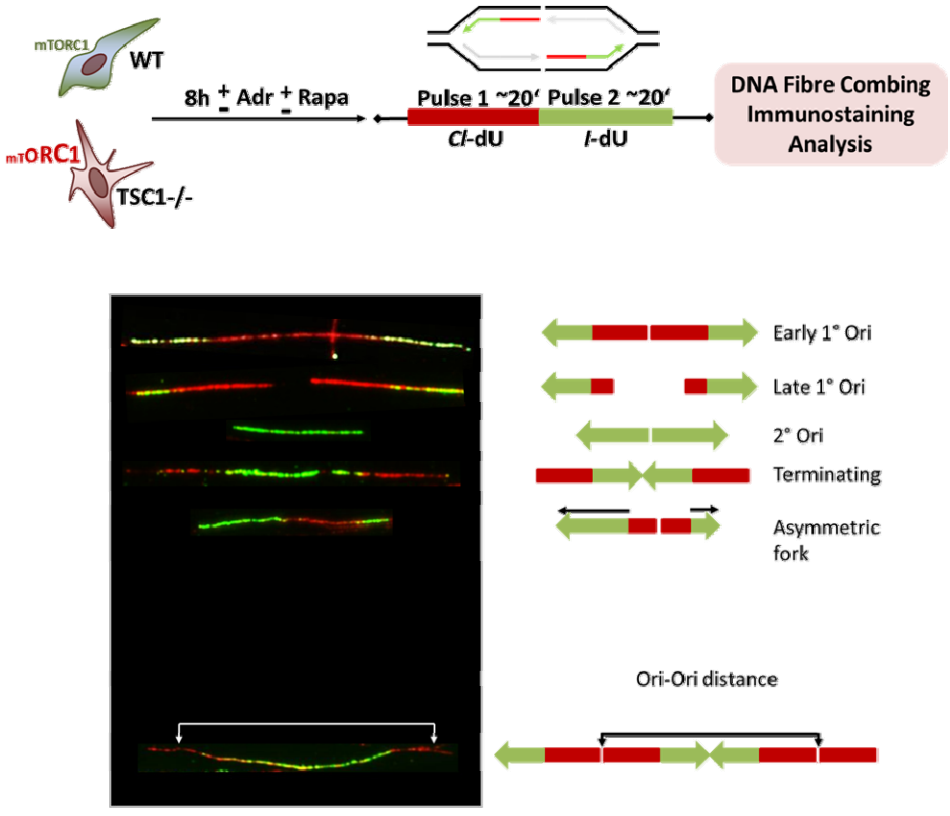


Figure 11: Protocol and representative replication structures/intermediates scorable by the DNA fibre assay.

Replication is the most vulnerable cell cycle phase predisposing cells to DNA damage and genetic instability and the fibre assay is the most reliable 'direct' readout of cellular S-phase events. Dual pulse labelling of DNA can be exploited to distinguish between several structural parameters for e.g., Inter-Origin distances, asymmetric forks, elongation or synthesis rates etc., some representative ones are shown above.

3.2.7.5. CFSE dye distribution/fluorescence decay for doubling time.

The cell-permeant fluorescein-based dye Carboxyfluorescein succinimidyl ester (CFSE) covalently labels long-lived intracellular molecules with the fluorescent dye carboxyfluorescein, resulting in uniform bright fluorescence. Upon division of CFSE-labelled cells, their progeny are endowed with half the number of carboxyfluorescein-tagged molecules (dye distribution) successively. Measuring the corresponding decrease in fluorescence intensity allows the resolution of daughter cell populations for over eight cycles of cell division by flow cytometry. The stable fluorescence and low cytotoxicity of CFSE, along with its compatibility with a broad range of other fluorochromes renders it suitable for multi-colour flow cytometry studies, particularly useful in determining division-related phenotypic and functional changes of cells, for instance- during B- and T-lymphocyte differentiation. Briefly, cells were washed twice with 1X PBS to eliminate protein contaminants from the serum and fed with 5µM CFSE in PBS for 15min followed by one PBS wash and replenishing growth medium. The 0h sample was collected about 4h post-feeding to allow sufficient reaction-time for the covalent coupling of intracellular structures with Carboxyfluorescein. Staining was performed in 6-well plates, all at once and samples collected and fixed in 4 % paraformaldehyde every subsequent day at a fixed time. At the end of 5 days, samples were washed once in PBS, and analysed by flow cytometry by plotting CFSE fluorescence against count. Successive peaks with diminishing fluorescence indicated sequential rounds of cell divisions. The doubling times were quantified by plotting median CFSE intensities for each sample against time in hours with the help of a previously created excel template.

4. Results

4.1. Work plan

The major aim of this project was to uncover the role of mTORC1 signalling as a susceptibility factor for genotoxic stress-induced cell death within the framework of the graduate programme and research training group RTG1715-molecular signatures of adaptive stress responses. Funded by DFG, the collective focus of projects within the graduate programme was to understand signalling mechanisms mediating cellular responses to a wide variety of external stressors in the context of hormetic and adaptive phenomena. The current project (SP15) was assigned to the core research area (CRA4) genotoxic stress response. A detailed description of the participating projects and research groups is available *on http://www.grk1715.uni-jena.de/*

Briefly, we set out to investigate whether high mTORC1 activity in TSC1-/- MEFs predisposes them to DNA damage by genotoxic cues. We further addressed cell cycle regulatory anomalies driven by aberrant mTORC1 signalling, and their role in sensitizing TSC1-/- MEFs to sub-lethal genotoxic stress. Finally, we also questioned whether this scenario might also be influenced by the impoverished energetic milieu, a hallmark of cells with high mTORC1 activity for e.g., as a requisite for DNA synthesis, surveillance and damage repair systems. Our tool kit to modulate mTOR activity included genetic (TSC1-/- MEFs, siRNA-mediated knockdown of TSC2 in WT MEFs), metabolic (amino acid depletion, calorie restriction) and pharmacological (mTOR inhibitors – Rapamycin, Torin1) to assess the resulting impact on cell viability over a backdrop of mild genotoxic stress.

4.2. Characterisation of the cell lines used for the study

Four cell lines derived from early stage embryos of WT, TSC1-/- , p53-/- and TSC2-/- p53-/- mice (C57BL/6J-BALB/cJ mixed background) henceforth MEFs for mouse embryonic fibroblasts, all obtained from Prof. David J Kwiatkowski's lab at Harvard University, Boston, MA, USA were used for the study. The Cell lines maintained under standard culture conditions were initially characterised for their mTOR pathway activity and Rapamycin sensitivity to establish the system and working model. The TSC1-/- MEFs and TSC2-/- p53-/- MEFs proliferated comparatively faster and clearly displayed elevated phosphorylation and activation states of S6 kinase, a direct downstream substrate of mTORC1 in Rapamycin-sensitive manner, and a negative IRS1 feedback-mediated Akt inhibition, consistent with literature. We have employed TSC1-/- and its counterpart TSC1+/+ (designated WT) MEFs for our experiment. Experiments were cautiously performed at early passages (p3-p5) although no difference between early and late passages was observed or reported.

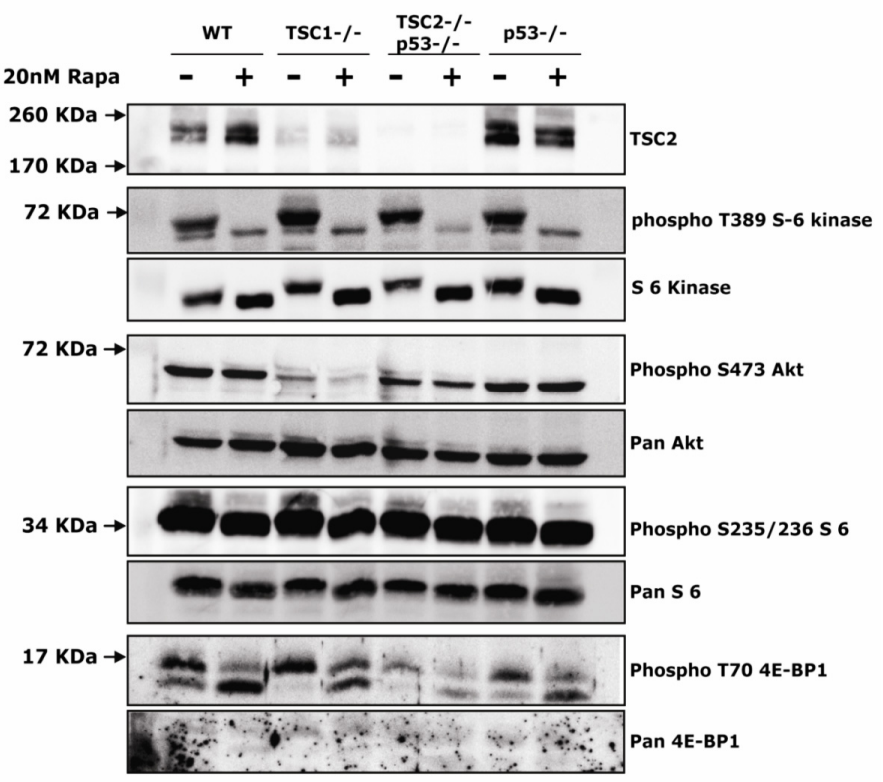


Figure 12: Western blot for characterisation of the cell lines used.
Note that the heterodimeric TSC complex is unstable in the absence of either partners -TSC1 or TSC2. The rapamycin sensitivity of mTORC1 activity is evident from S6K phosphorylation at T389, also the feedback inhibition on Akt in TSC1-/- and TSC2-/-p53-/- MEF lines. Others have observed a mobility shift in S6K upon phosphorylation, also seen here. 4EBP1, another key downstream target of mTORC1 is only partially inhibited by rapamycin treatment.

4.3. TSC1-/- loss sensitizes cells to mild genotoxic stress-induced death

Firstly, we placed TSC1-/- MEFs under acute and extended treatments with variety of metabolic and genotoxic stressors, to screen for general viability under these growth conditions. Picking on subtle doses, well tolerated by their WT counterparts, we observed that, in line with previous reports, subjecting TSC1-/- MEFs to sub-lethal metabolic and genotoxic stress results in precipitous detachment and rounding off, indicating loss of cell viability. Glucose deprivation or treatment with low doses methyl methane sulphonate (MMS, an alkylating, inter-strand crosslinking agent), Hydroxyurea (HU, a ribonucleotide reductase inhibitor) or Adriamycin (Adr or Doxorubicin, an intercalating chemotherapeutic causing double strand breaks), all resulted in massive dying of TSC1-/- cells within 20hrs (Figure 13A, 13B and 13C). Guan et.al., have in a seminal paper, described such hypersensitivity of TSC1-/- MEFs to acute glucose starvation and to sub-lethal doses of the alkylating agent MMS, two distinct forms of stress – metabolic and genotoxic (Lee et al., 2007). For our experiments, we chose to test the outcome of common chemotherapeutics, a single strand breaking agent (Hydroxyurea – HU) and a double strand breaking agent (Adriamycin – Adr, a.k.a. Doxorubicin), at virtually low doses for our experiments. HU inhibits ribonucleotide reductase, an enzyme catalysing the conversion of ribonucleotides to deoxyribonucleotides, thus depleting the intracellular deoxy-nucleotide pools and causing replication blockade, single strand gaps and breaks. Adr is chiefly an anthracyclin antineoplastic which intercalates with DNA strands. Certain undesirable minor effects of both drugs have been described, particularly with longer treatments. Although detachment from culture substratum is a sign of cell death, Figure 13C eliminates any transient cell cycle arrest, if at all, by confirming the loss of membrane integrity (red staining with impermeable dye Propidium Iodide). Thus TSC1-/- cells are indeed hypersensitive to mild stress-doses *in vitro*. We then attempted to quantify cell death by multiple approaches since it was necessary to understand the nature and magnitude of such stress-induced cell death in TSC1-/- MEFs and furthermore, to verify if mTORC1 inhibition in TSC1-/- MEFs can confer protection from stress-induced death.

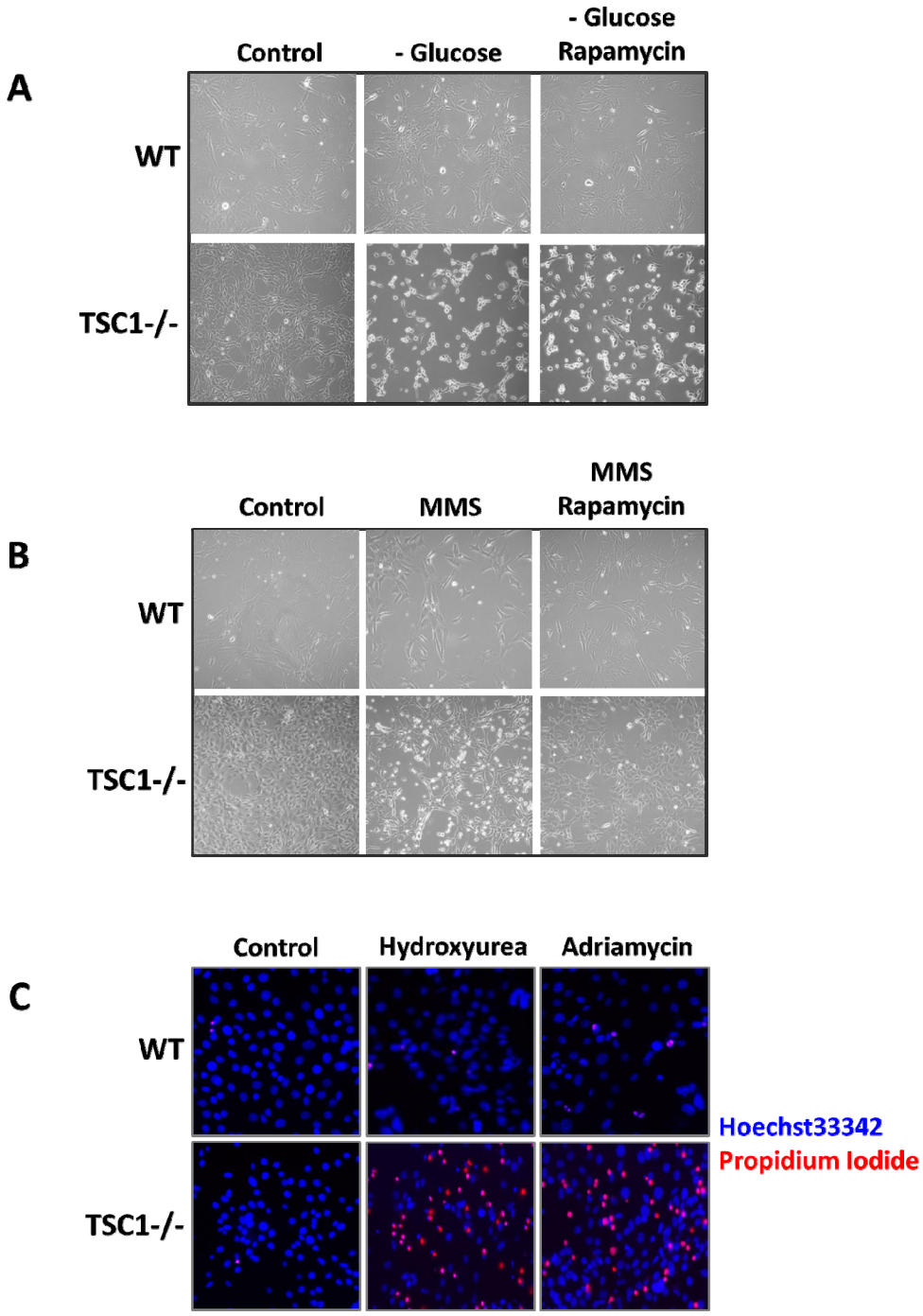


Figure 13: TSC1-/- MEFs are hypersensitive to acute metabolic and sub-lethal genotoxic stress.
A) Light microscopy of WT and TSC1-/- MEFs untreated or subjected to glucose deprivation for 20h, in the presence or absence of mTORC1 inhibitor Rapamycin (20nM). **B)** Light microscopy of WT and TSC1-/- MEFs untreated or treated with the alkylating agent Methyl Methane Sulphonate (MMS, 25µg/mL) for 20h, with or without Rapamycin. **C)** Fluorescent microscopy WT and TSC1-/- MEFs untreated or treated with Hydroxyurea (HU, 2mM) and Adriamycin (Adr, 0.5µg/mL) respectively for 20h. Hoechst33342 (membrane permeable, live-cell nuclear stain) and Propidium iodide (membrane impermeable dead cell stain) mark live and dead cells. Note the abundance of floating cells (A and B), and PI-positive, red-stained cells (C) indicating dying of TSC1-/- MEFs in response to mild stress. Note that rapamycin appears to confer protection to MMS-induced death of TSC1-/- MEFs.

MTT formazan dye-conversion viability assays, AnnexinV-labelling flow cytometry and propidium iodide (PI) membrane exclusion assays were all performed to gauge the nature and magnitude of cell death in TSC1-/- MEFs. However, the PI exclusion assay proved to be a reliable readout for cell death and our results yielded an exacerbated death toll in TSC1-/- MEFs under mild genotoxic stress in comparison to WT MEFs. Acute treatment of TSC1-/- cells in growth media with sub-lethal concentrations of genotoxic agents (or simply withdrawing glucose, data not shown here, reported elsewhere) led to precipitous cell death (Figure 14A).

Performing western blots after identical treatments, we found that the genotoxic death in TSC1-/- MEFs correlated with classical DNA damage response (DDR) activation, as evidenced by p53 activation (pSer15 p53) and Poly ADP–Ribose Polymerase (PARP) cleavage, an early event in programmed cell death (Degterev & Yuan, 2008; Dorn, 2013; Elmore, 2007). A general upregulation (abundance) and stabilisation of p53 upon DNA damage has been attributed to the death phenotype observed in TSC1-/- MEFs (Lee et al., 2007). We noted that while WT MEFs needed a much stronger insult even for modest induction of p53, its elevated levels in TSC1-/- MEFs appeared to signal DNA-damage and apoptosis/cell death (Figure 14B), although at this stage it was not possible to pinpoint the exact mechanism of death. However, whether these cells attempt a cell cycle arrest before initiating death programmes, and whether other factors for instance, inadequate energy for restorative processes played a role was a matter of further investigation, particularly in view of the poorly understood cell cycle progression of TSC1-/- cells. mTORC1 over-activity has been linked to increased anabolic energy consumption and diminished energy levels in TSC-/- cells. Others have reported (R. T. Abraham & Eng, 2010; Choo et al., 2010) and we have consistently observed higher AMP-activated kinase (AMPK) activity and lower ATP levels in these cells, addressed in more detail in section 4.9.

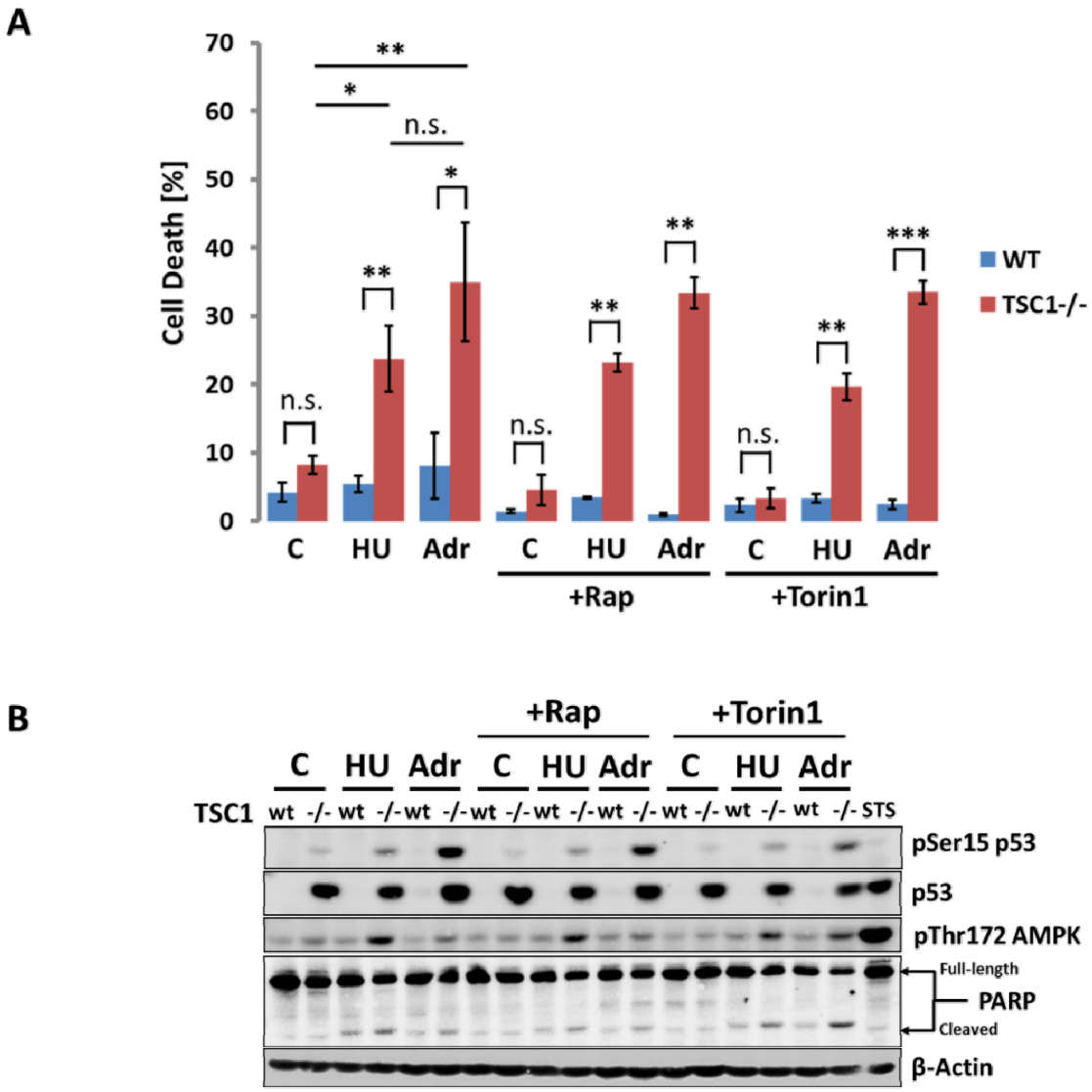


Figure 14: Sub-lethal genotoxic stress, DNA damage response and cell death.
A) Propidium iodide exclusion flow cytometry for cell death quantification. WT and TSC1-/- MEFs untreated or treated with Hydroxyurea (HU, 2mM) and Adriamycin (Adr, 0.5µg/mL) respectively for 20h. Wherever indicated, mTORC1 Inhibitors Rapamycin (Rap, 20nM) or Torin1 (10nM) were spiked 2h prior to genotoxic treatments. Exalted cell death in TSC1-/- MEFs is evident. Data-set are a mean of duplicate samples from three independent experiments. Error bars represent standard deviation (SD). One-way ANOVA (for group comparisons, straight lines) and the two-tailed t-Test (for pair-wise comparison, brackets) was used for statistical analysis. *P<0.05, **p<0.01, ***p<0.001, n.s.- not significant. Pair-wise significance is as indicated (WT vs TSC1-/-). Significance indicated for the inhibitor-free group is applicable to rapamycin and torin1 treatments as well; not shown here for reasons of clarity. **B)** Representative western blot of WT and TSC1-/- MEFs treated as in A. Note the elevated levels of p53 in TSC1-/- MEFs. TSC1-/- cells appear to initiate classical DNA damage response pathways – p53 activation and PARP cleavage following genotoxic treatments. Also note the generally higher activation states of AMPK implying an increased energy demand in TSC1-/- MEFs. STS- Staurosporine used as a positive control for detection of cleaved PARP.

Furthermore, TSC1-/- cells under genotoxic treatment (HU and Adr) not only have increased Caspase-3 and PARP cleavage, but also initiated caspase-3 cleavage at significantly lower doses (Figure 15A) compared to their WT counterparts. In conjunction with the AnnexinV labelling flow cytometry experiments (not shown here) these results evidence apoptosis; however, this accounts for only a fraction of the dying cell population as PI exclusion assays projected a significantly higher overall death toll, suggesting there may be other non-apoptotic mild genotoxic stress-induced death programmes operating in TSC1-/- cells. Upon verifying whether this observation is solely due to the single gene loss (TSC1-/-) and is not confounded by high rates of mutations in culture resulting in mal-adaptations, we found that an acute siRNA-mediated knockdown of TSC2 essentially recapitulated salient features of TSC1-/- MEFs with regard to their signalling pattern and stress sensitivity (Figure 15B).

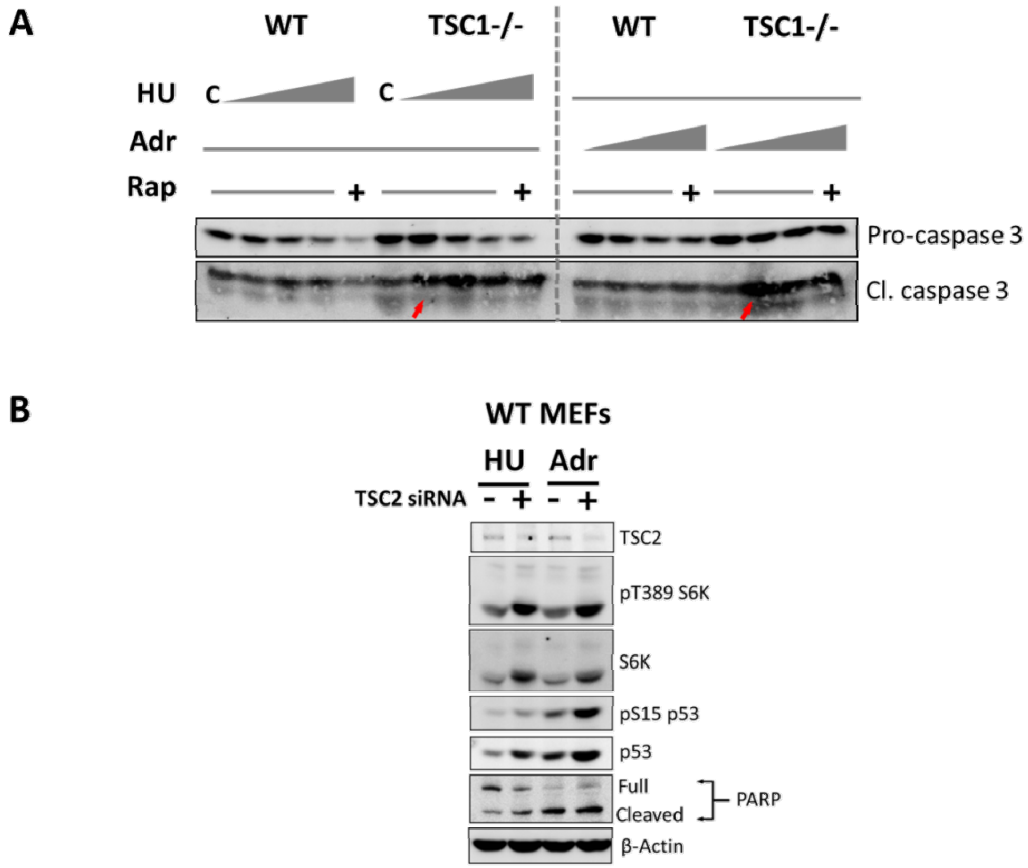


Figure 15: TSC1-/- cell death is partly apoptotic and TSC loss alone is sufficient to sensitise cells to mild stress.

A) Western blots displaying caspase-3 cleavage and apoptosis initiation in TSC1-/- MEFs in response to mild genotoxic stress. Note the accumulation of cleaved Caspase-3 at much lower doses in TSC1-/- MEFs with both HU as well as Adr (red arrowheads), implying hypersensitivity to stress. A certain fraction of all dying TSC1-/- cells undergo apoptosis, since PI exclusion estimated a higher overall death count. **B)** siRNA-mediated acute knockdown of TSC2 in WT MEFs recapitulates several features

of TSC1-/- genotype. Cell lines maintained for long periods of time can rewire their signalling, accumulate mutations in vitro, and readapt. To eliminate effects not due to loss of TSC function (mal-adaptations), we knocked down TSC2 in WT MEFs, essentially recapitulating characteristics observed in the TSC1-/- cells. Note the constitutive S6K phosphorylation, increased p53 expression and activation and PARP cleavage, all reminiscent of the TSC-/- genotype.

4.4. TSC1-/- cells accumulate excessive DNA damage in response to mild genotoxic insult

DNA damaging agents are known to affect rapidly proliferating cells more than normal cells. On an average, p53 is mutated in about 50% of all cancers (Lane & Levine, 2010; Soussi & Wiman, 2007; Vogelstein, Lane, & Levine, 2000) and ranges from 6-48% in various sporadic cancers (Goldstein et al., 2011; Olivier, Hollstein, & Hainaut, 2010). TSC1-/- MEFs however, accumulate and stabilise p53 in response to mild genotoxic insult. We wondered if loss of viability of TSC1-/- MEFs under such conditions is due to an exacerbated response to the mild stress dose or whether they accrue higher levels of primary genetic insult. Using multi-colour flow cytometry for cell cycle analysis we found that TSC1-/- MEFs accumulated higher amounts of DNA strand breaks (γH2A.x readout) in the presence of otherwise harmless doses of genotoxic stress. About 75% of all TSC1-/- cells were damaged (γH2A.x-possitive) compared to 18% in the WT cells within 20h of low-dose drug exposure (Figure 16A, left). Dose-response westerns and densitometry confirmed that TSC1-/- cells were prone to higher genetic insult at low doses and the peak phosphorylation levels of γH2A.x correlated with the concentration and treatment-time at which they succumbed to death (Figure 16B and 16A, right). Such a “tipping-point” in cellular decision-making resulting in an inverse, non-linear outcome to a linear increase in stress-dose has been termed “hormesis” and is a matter of intense investigation (Calabrese & Baldwin, 2001; Gems & Partridge, 2008; Löffler et al., 2008). With these fundamental set of results, we corroborate our initial hypothesis, that such drastic loss of adaptive capacity in TSC1-/- cells, and a skewed hormetic curve (refer to hypothesis and aims) is due to the derangement of the mTORC1 pathway, thus predisposing them to mild stress-induced death.

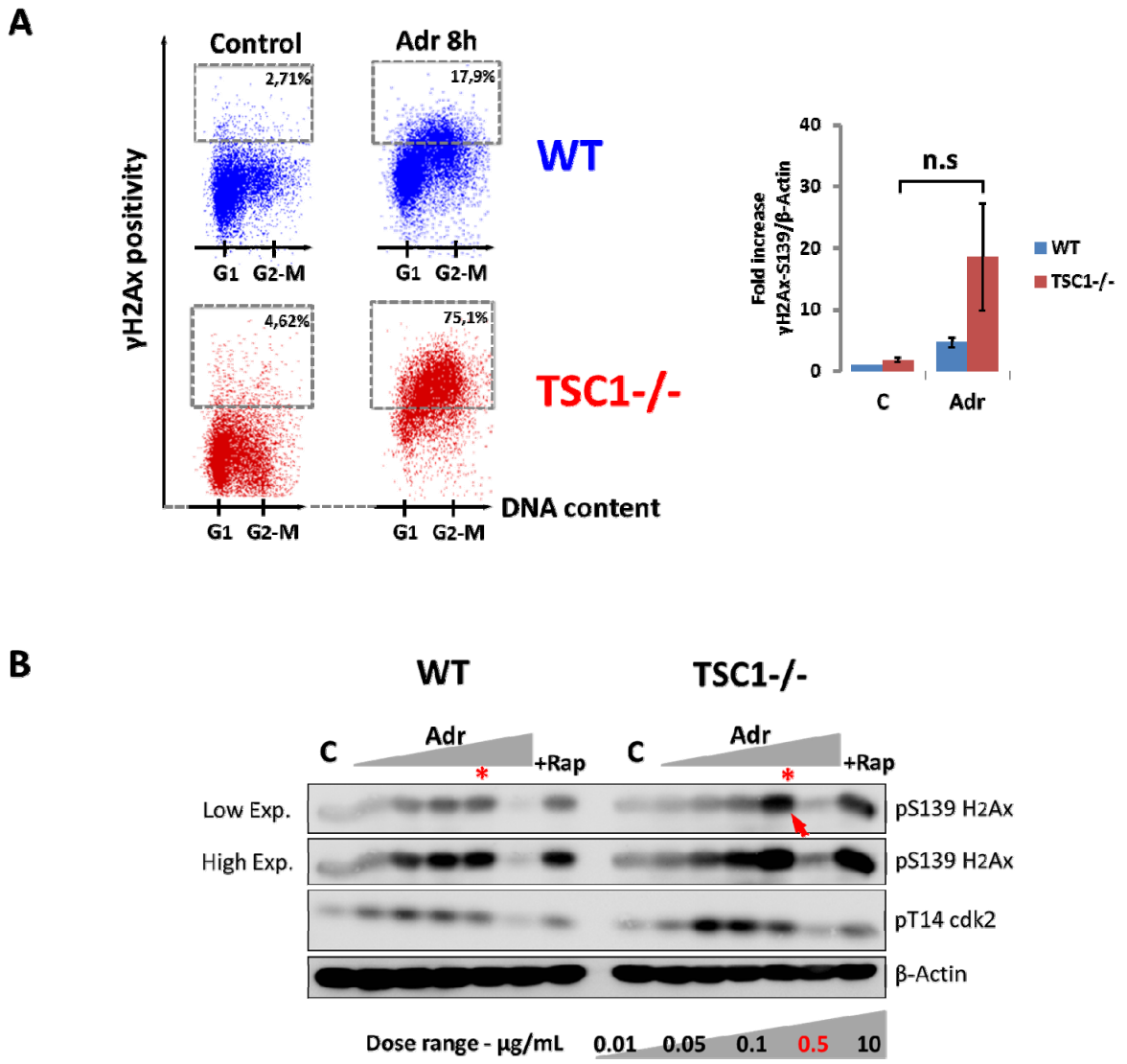


Figure 16: High mTORC1 activity predisposes TSC1-/- MEFs to profuse DNA damage.
A) (Left) Representative dot plots of γH2A.x(Ser139)/propidium iodide flow cytometry for DNA damage estimation in WT and TSC1-/- MEFs, untreated or acutely treated with 0.5µg/mL adriamycin for 8h. Over 75% of all TSC1-/- MEFs are γH2A.x-positive, indicating that they are prone to strand breaks and accrue much higher damage levels under mild Adriamycin stress. (Right) Densitometry of γH2A.x(Ser139) western blots from 3 independent experiments indicating higher phosphorylation levels in TSC1-/- MEFs after both acute-8h and longer-20h adriamycin treatment. Values are mean ± SD. Statistical significance was calculated using two-tailed t-Test. n.s.-not significant. **B)** Dose-response westerns of WT and TSC1-/- MEFs treated for 20h with the indicated dose-range of Adriamycin. Notice the drastic accumulation of γH2A.x in the 0.5µg/mL Adriamycin-treated TSC1-/- sample, which well correlates with the cell death observed in the PI exclusion assays. +Rap indicates 0.5µg/mL Adriamycin along with rapamycin.

4.5. TSC1-/- cells display alterations in their cell cycle distribution and doubling time

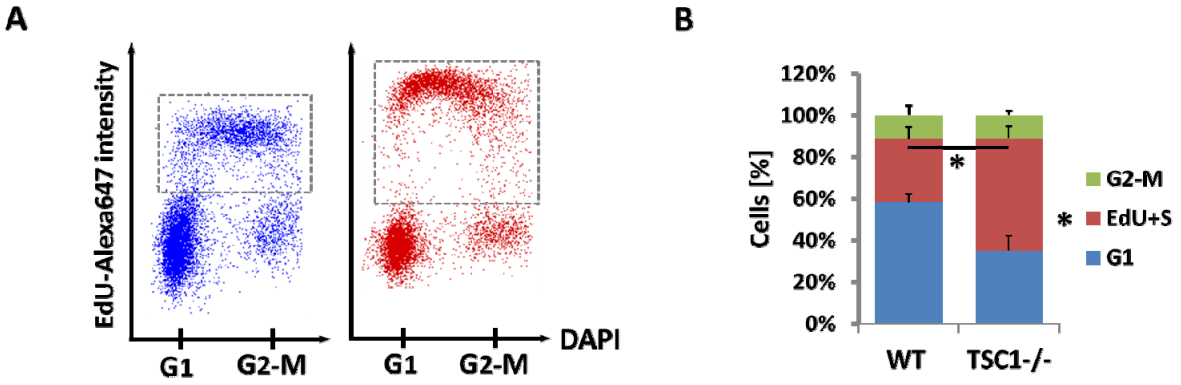


Figure 17: TSC1-/- MEFs have an altered cell cycle distribution.
A) EdU incorporation cell cycle profiles of untreated WT and TSC1-/- MEFs; the S-phase arcs clearly differ, in that, the height of the TSC1-/- arc surpasses that of the WT MEFs, indicating faster incorporation of the base analogue given a 15 min EdU pulse **B)** Average distribution of cells in various cell cycle phases. Notice the higher proportion of S-phase and a corresponding decrease in G1-phase TSC1-/- cells. Values are mean ± SD of three independent experiments. Statistical significance was calculated using two-tailed t-Test. *p<0.05.

mTORC1 is a pro-anabolic growth promoting pathway and an important feature of cells with constitutive mTORC1 signalling is their frail cell cycle checkpoint regulation. High mTORC1 activity is known to shorten G1-phase, by accelerating G1-to-S phase transition and/or premature S-phase entry (Hengstschläger et al., 2001; Hengstschläger & Rosner, 2003; Wang & Proud, 2009). To investigate whether loss of TSC1-/- alters cell cycle distribution and progression, we performed (EdU) pulse-incorporation followed by click chemistry-based fluorescent labelling and flow cytometry (together with DAPI and mitotic marker pSer10-$HisH_3$, multi-colour). We observed an expected increase in S-phase population of TSC1-/- cells and a compensatory decrease of G1-phase cells under various growth conditions. This was accompanied by significantly higher peak EdU incorporation (DNA synthesis) rates characteristic of transformed/tumour cells (Figure 17A and 17B).

Furthermore, CFSE dye dilution approach to measure doubling time, and deriving the time spent in each cell cycle phase from their distribution, yielded a rough indication of a nearly 50% shorter G1-phase and a 1.5 fold longer S-phase in TSC1-/- MEFs, suggesting premature S-phase entry. However, the overall higher EdU incorporation rates and the apparently longer S-phase were contradictory and intriguing. DNA replication should in principle be

completed faster in case of higher incorporation rates, as is evident in cancer cells. More detailed inspection of the flow cytometric cell cycle plots suggest that TSC1-/- cells show particularly high EdU incorporation, whereas the same cells apparently synthesise DNA more slowly in late S-phase, which may explain the increase in S-phase population. This urged us to take a closer look at cell cycle progression; we then performed time-course experiments after Adriamycin treatment.

4.6. TSC1-/- cells have frail cell cycle regulation under mild genotoxic treatments

To understand cell cycle regulation under basal conditions and in presence of mild genotoxic stress, we followed WT and TSC1-/- MEFs in time after Adriamycin treatment in similar Pulse-labelling EdU/DAPI/pSer10-HisH3 flow cytometry experiments. We were particularly interested in the S-phase events, since it is the most vulnerable cell cycle phase predisposing cells to genetic alterations and instability leading to damage-induced cell death or cancer (Halazonetis, Gorgoulis, & Bartek, 2008; Osborn et al., 2002). Several revelations caught our attention.

Firstly, untreated TSC1-/- MEFs displayed elevated EdU incorporation rates and the mean, median and mode of the EdU-Alexa647 fluorescence intensity was roughly 1.5-1.7 fold higher than their WT counterparts (Figure 18A). Next, despite higher damage accumulation, TSC1-/- MEFs displayed consistent, unabated EdU incorporation arcs under adriamycin treatment as opposed to a tangible decline in the WT MEFs after 4-8h of treatment (Although minor, notice the tilt in the EdU arcs in WT samples marked with a red arrow, possibly indicative of a decline in DNA synthesis to allow repair processes). Lastly, while there were negligible differences at intermediate time points including 12 h and 16h, TSC1-/- cells culminated with a catastrophic S-phase and a massive G2-M accumulation in comparison to the WT MEFs (Figure 18A, at 20h Adr, red arrow, quantification lower panel), and continued adriamycin treatments beyond 24h abolished re-population of G1-phase in both cell types, meaning that the G2-M arrest did not permit entry into subsequent cell division cycles (not shown here) in the presence of low-dose adriamycin.

Attempts to initiate DNA damage responses via p53 activation (pSer15 p53) and impose cell cycle arrest via cdk inhibition (pThr14 cdk2) by TSC1-/- MEFs under Adriamycin treatment seems to remain unsuccessful in backdrop of p53 overabundance and Cdk2 downregulation. Moreover, cdc45 appears to diminish with increasing exposure to adriamycin, suggesting that its function within the Cdc45-Mcm2-7-GINS or CMG helicase complex (Aparicio, Ibarra, & Mendez, 2006) may be affected under stress in TSC1-/- MEFs (Figure 18B and 18C; see also Figure 19C, synthesis rates). Altogether, high mTORC1 activity, directly or indirectly, renders TSC1-/- cells resilient to cell cycle arrest, despite DNA damage and lowered synthesis rates.

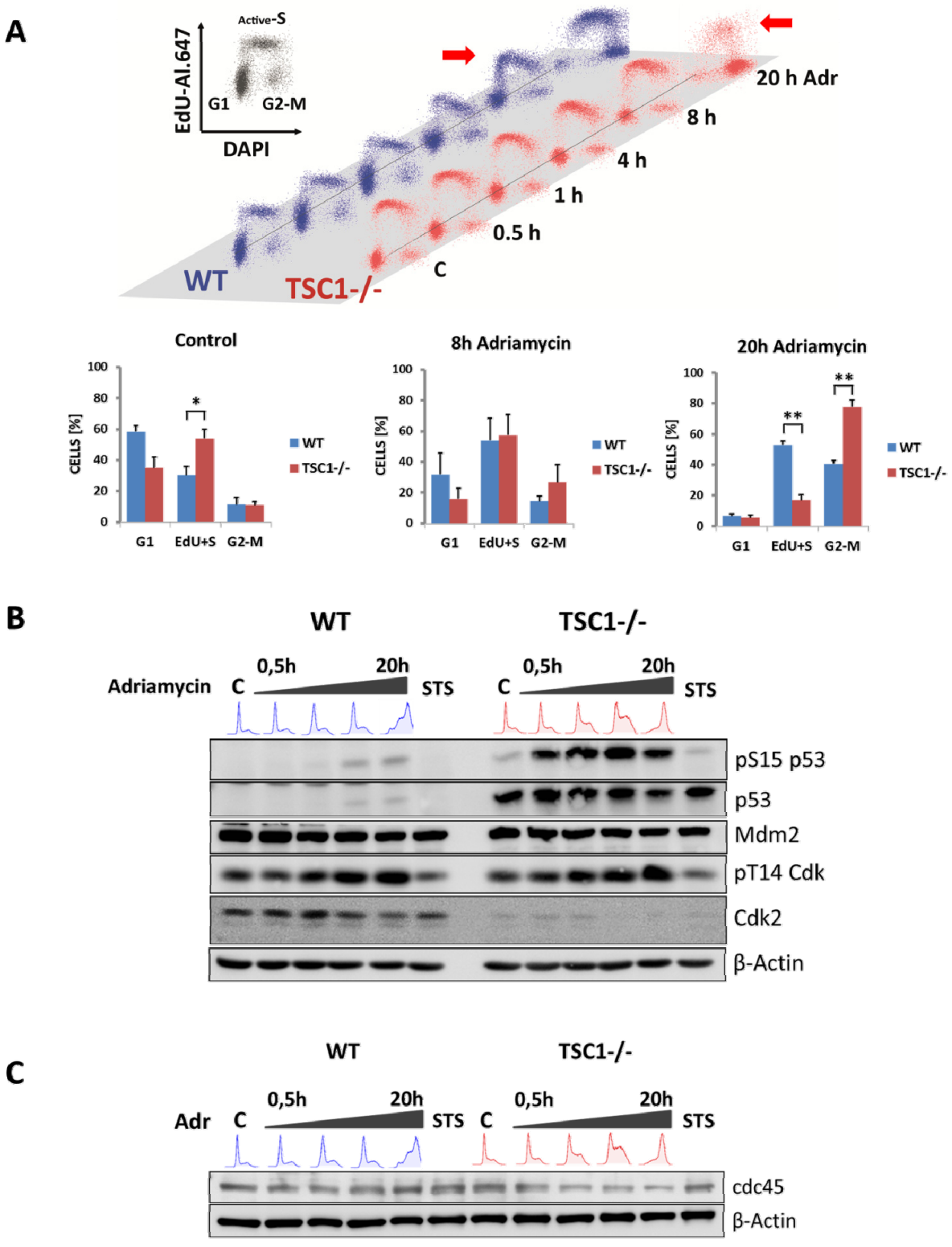


Figure 18: TSC1-/- MEFs are hypersensitive to acute metabolic and sub-lethal genotoxic stress.
A) (Upper panel) Representative kinetic EdU incorporation cell cycle profiles of WT and TSC1-/- MEFs following 0.5µg/mL adriamycin treatment for indicated periods of time, and (lower panel) average distribution of cells in various cell cycle phases at the indicated time points. Observe the decline in incorporation in late S-phase at 8h in WT MEFs (red arrow) compared to the incessant incorporation rates in TSC1-/- MEFs, seemingly unperturbed by the presence of the drug. Also notice the catastrophic S-phase arc in TSC1-/- MEFs at 20h and the massive G2-M arrest (lower panel). Over

75% of the cells pass through S-phase and accumulate in G2/M. Values are mean + SD. Statistical significance was calculated using two-tailed t-Test. *p<0.05, **p<0.01. **B)** Western blot of WT and TSC1-/- MEFs treated as in A (1h time-point omitted). TSC1-/- cells appear to initiate damage and checkpoint responses under Adriamycin treatments by activating p53 activation and inactivating Cdk2. The repressed Cdk2 expression levels, in addition to the elevated p53 levels in TSC1-/- MEFs are noteworthy. STS – Staurosporine. **C)** Western blot for cdc45 in WT and TSC1-/- MEFs treated as in Figure 17A, 17B. cdc45, within the CMG complex comprising of MCM2-7 and GINS, termed the 'unwindosome', plays an important role in replication initiation and elongation.

4.7. TSC1-/- cells experience a replication phenotype, distinct from classical replication stress

Eukaryotic genomes have on an average a potential origin every 10 kb and about one active origin every 100 kb; in that respect, the human genome has 30,000 – 40,000 active origins of replication (Jackson & Pombo, 1998). Potential origins, called dormant origins are licenced and lie interspersed operating only to rescue stalled or collapsed forks by offering a possibility of continuing synthesis from a neighbouring site in the event of unrepaired lesions or S-phase stress (Ge, Jackson, & Blow, 2007). A primeval event in tumorigenesis is oncogene activation (Halazonetis et al., 2008). Activated oncogenes, in an attempt to drive rapid proliferation, accelerate DNA synthesis. A direct consequence of this is replication stress, often due to over-firing or exhausting dormant (rescue) replication origins. A striking feature of TSC1-/- MEFs, the observation that they have higher overall DNA synthesis rates (EdU incorporation intensities) even in the presence of DNA damage led us to believe that they may be experiencing a replication phenotype driven by constitutive mTORC1 activity (Figure 19A). It is conceivable that 'premature' entry and hasty progression through S-phase may, via erroneous processes like surplus replication origin firing and intra-S checkpoint overriding, facilitate the accumulation of potentially lethal DNA damage in the presence of otherwise harmless genotoxic stress. We thus performed DNA fibre assays to inspect S-phase progression in WT and TSC1-/- MEFs treated with Adriamycin for 8h, following the observation that WT MEFs curtail incorporation rates at least in the late S-phase to cope with the DNA damage while TSC1-/- cells synthesised incessantly.

DNA damage–response and replication checkpoint kinases ATR and Chk1 coordinate a critical genome surveillance network allowing cells to cope with replication stress and DNA damage (Lecona & Fernandez-Capetillo, 2014). On one hand, they are frequently

upregulated in cancers addicted to maintenance of a proficient RS-checkpoint to tolerate RS and ensure survival; while on the other hand, the checkpoint responses are 'dampened' to allow arrest-free rapid proliferation of cancer cells (Zeman & Cimprich, 2014). We observe that in TSC1-/- MEFs, while ATR is substantially down-regulated and Chk1 levels unaltered, their activation states are comparable to WT MEFs under Adriamycin treatment indicating proficiency of the upstream ATR-Chk1 RS-response (Figure 19B). Findings from the fibre assays revealed that TSC1-/- MEFs have slower fork progression rates despite a globally increased incorporation rate under basal conditions. This ambiguity of high overall incorporation rates vs. slower strand elongation rates at sole origins pointed to elevated origin firing. In fact, Ori-Ori distances in TSC1-/- MEFs are ~15% lower compared to WT MEFs, suggesting moderate over-use of origins; the lower synthesis rates possibly due to distribution of nucleotide pools among greater number of origins as well as a general energy short-supply in TSC1-/- MEFs (AMPK activation, ATP levels). A marked deviation from classical RS phenotype is the absence of spontaneous fork asymmetry, a hallmark of augmented replication fork stalling, although Adriamycin treatment generated DNA damage and elevated asymmetry in TSC1-/- MEFs (Figure 19C). We believe TSC1-/- MEFs experience a constitutive mTORC1-driven subtle, *ATP-restricted replication phenotype*, rendering them highly sensitive to mild stress-induced cell death.

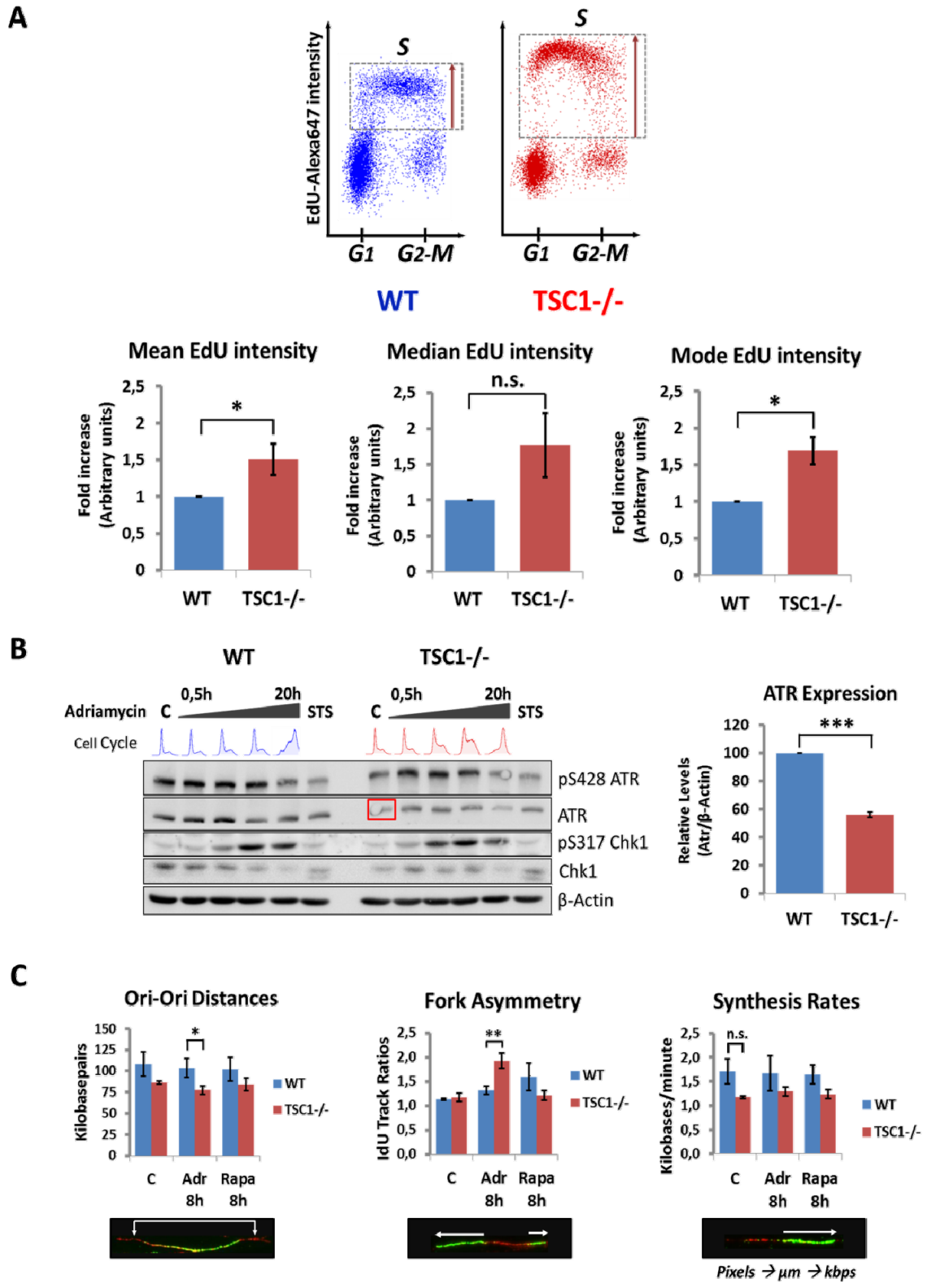


Figure 19: TSC1-/- MEFs are prone to a replication stress-like phenotype

A) (Upper panel) EdU incorporation Cell Cycle profiles of untreated WT and TSC1-/- MEFs, as in Fig.17, portraying higher EdU arcs in TSC1-/- MEFs, and (lower panel) the mean, median and mode of the EdU-Alexaflour647 fluorescence intensities indicating higher overall DNA synthesis. **B)** (Left) Western analysis of replication checkpoint kinases Atr and Chk1 of WT and TSC1-/- MEFs treated as in Fig.17. Although there appears to be a marginal, yet general down-regulation of ATR, the

phosphorylation of both ATR and Chk1 in response to adriamycin indicates RS-checkpoint proficiency. (Right) Atr densitometry of untreated WT and TSC1-/- MEFs from six independent experiments at low and high exposures. Chk1 levels in TSC1-/- MEFs are unchanged. The downregulation of ATR level is arguably an RS-tolerance and cell cycle arrest evasion mode adopted by transformed cells. **C)** Panel shows inter-origin distances (Kb), replication fork asymmetries (IdU track ratios) and average DNA synthesis rates per origin (kb/min). Although spontaneous fork asymmetry, a classical feature of RS, is completely absent in TSC1-/- cells, significantly higher asymmetry was observed within 8h of adriamycin treatment. The lower fork progression (or synthesis) rates and shorter inter-origin distances in TSC1-/- MEFs suggest modest origin over-firing and diminished nucleotide or energy supply at replication factories. Bars indicate mean ± SD. Statistical significance was calculated using two-tailed t-Test. *p<0.05, **p<0.01, ***p<0.001, n.s.- not significant.

4.8. TSC1-/- MEFs appear to have a leaky ATM-Chk2 mediated G2-M checkpoint

The G2/M checkpoint prevents mitotic entry of cells with under-replicated or damaged DNA. While the G2/M checkpoint is predominantly governed by the ATM-Chk2 pathway (Fernandez-Capetillo et al., 2002; Kousholt, Menzel, & Sorensen, 2012; Stark & Taylor, 2004), the ATR kinase is known to coordinate chromosome condensation with nuclear envelope breakdown (Lecona & Fernandez-Capetillo, 2014). In the light of ATR downregulation, and since we observed exalted cell death (PI exclusion data) and a catastrophic S-phase population accompanied by massive G2-M arrest (EdU FACS data) at 20h in TSC1-/- MEFs, we questioned the possibility of a mitotic catastrophe and pursued investigating the fidelity of the G2-M checkpoint control. Using a third staining for pSer10-HisH3 to specifically mark M-phase cells, in time-course experiments under Adriamycin treatment; it was possible to distinguish between G2 and M phase cells. While western blot analysis of Chk2 phosphorylation pattern indicated functional G2-M checkpoint responses, the ratio of G2 to M phase cells hinted at a leaky checkpoint in effect leading to promiscuous, damage-prone and mitotic entry of TSC1-/- MEFs (Figure 20).

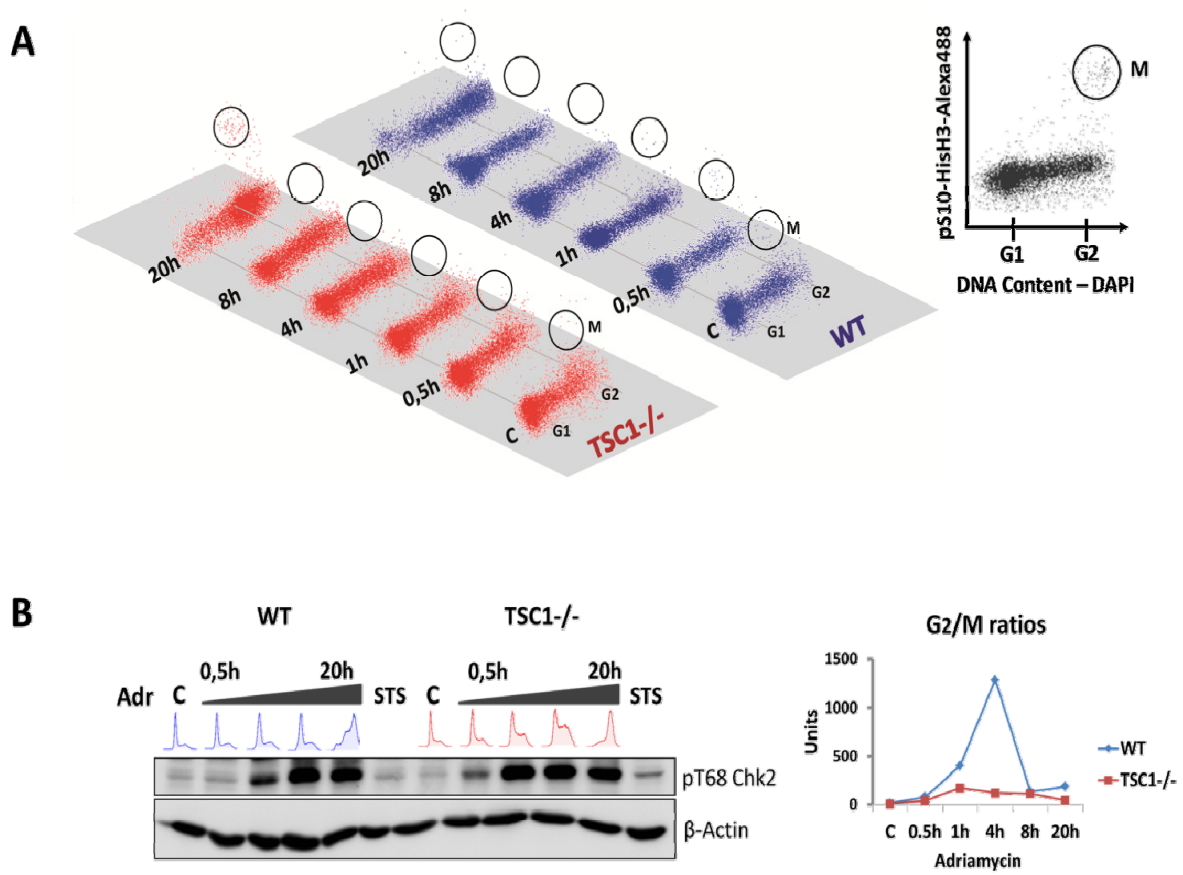


Figure 20: Mild stress-induced death of TSC1-/- MEFs may be attributed to an apparently leaky G2-M checkpoint.

A) Mitotic entry monitored at various time-points after 0.5µg/mL Adriamycin treatment in WT and TSC1-/- MEFs, by DAPI/pSer10–HisH3 flow cytometry. HisH3 phosphorylation at ser10 is a stringent mitotic event occurring at prometaphase triggering chromosome condensation. This has been exploited as a marker to distinguish between G2 and M phase cells by plotting DNA content versus pSer10–HisH3 (inset in black). Both cell lines initially respond to the stress by repressing mitotic entry, however, TSC1-/- MEFs display far more mitotic events at 20h, hinting that damage prone mitosis may be driving them to catastrophic death. **B)** (Left) Western blot showing chk2 activation, indicating a proficient ATM-Chk2-mediated G2-M checkpoint after adriamycin damage; (Right) Percentage ratios of G2 to M phase cells as a measure of the fidelity of G2/M checkpoint, plotted as the geometric mean of 2 experiments; the lower ratios in TSC1-/- MEFs suggest a checkpoint maintenance defect, eventually permitting damage-prone mitotic entry.

4.9. Metabolic and genotoxic stressors differentially impact TSC1-/- MEF viability

AMP-activated protein kinase (AMPK) is the master energy sensor coordinating cell growth with ATP sufficiency (Codogno & Meijer, 2005; Hardie, 2011; Laderoute et al., 2006). In line with previous reports, our attempts to characterise preferential energy source and

consumption revealed an overall higher ATP consumption in TSC1-/- cells under a diverse range of growth conditions suggesting that a general energetic insufficiency may exacerbate several readouts of genotoxic stress-induced death in our studies. Such trend of lower ATP levels is also concordant with AMPK activation states (pThr172 AMPK) in TSC1-/- cells (Figure 21A). L-Glutamine is known to be a strong source of energy and can be shunted into several biosynthetic pathways under conditions of starvation (Hensley, Wasti, & DeBerardinis, 2013). Glutamine 'anaplerosis' has been shown to protect tumour cells from starvation and metabolic stress-induced death; in fact Glutamine alone is known to sustain cell viability for extended periods of time *in vitro* (Chiu et al., 2012; Choo et al., 2010; Durán & Hall, 2012). We observe that in the absence of all other nutrient sources, L-Glutamine serves as a sustained energy source, while essential amino acids alone, result in a severe drop in ATP levels (Figure 21A).

We then sought to evaluate how energy limitation or availability influences the sensitivity of TSC1-/- cells to mild genotoxic stress. Hence we performed western analysis with a range of manipulations, 1. To inhibit mTORC1 pathway (Torin1), 2. Modulate ATP levels – Glucose deprivation/2-deoxy-glucose treatment and essential amino acids to deplete ATP and Glutamine as a high ATP substrate (as per Figure 21A) 3. Relieve the replication phenotype by nucleoside-feeding, thus excluding lower synthesis rates due to nucleotide shortage, all in the absence or presence of Adriamycin for 8h. We found that Nsd and GLn could relieve DNA damage (γH2Ax phosphorylation) while essential amino acids, by further augmenting protein synthesis, accelerated it indicating energy shortage synergises with genotoxic agents in causing damage (Figure 21B).

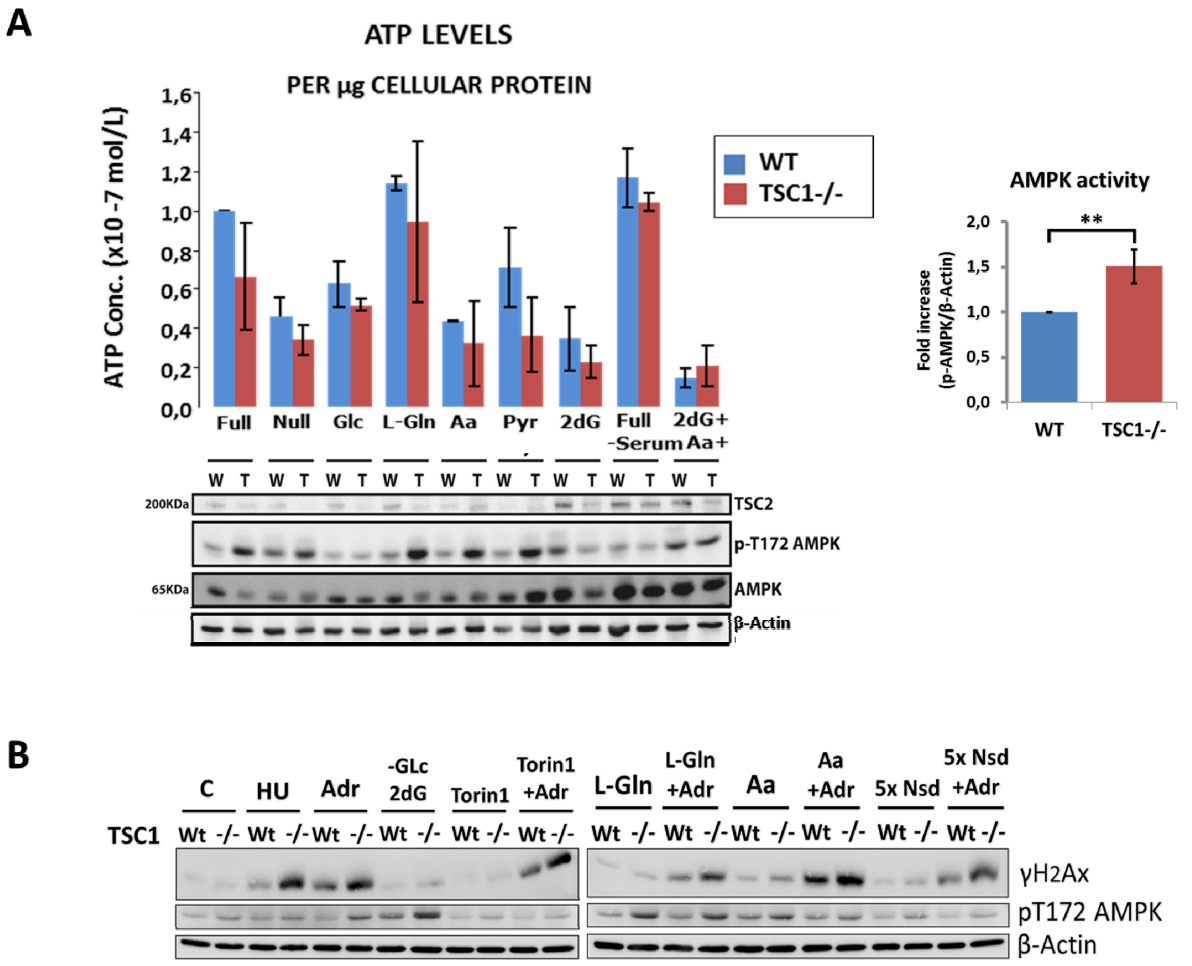


Figure 21: Energy deficit in TSC1-/- MEFs amplify DNA damage accumulation.
A) Left above – Luminometric ATP measurements of WT and TSC1-/- MEFs under diverse growth conditions as indicated for 20h. Left below - Western blot of duplicate samples showing concomitant AMPK activation states indicating an energetic overload on TSC1-/- MEFs. Glutamine anaplerosis is known to boost energy levels and cell function under conditions of glucose starvation/diminished ATP levels. Essential amino acids on the other hand appear to accelerate protein synthesis further by enriching intracellular pools resulting in a drastic reduction of ATP levels, in the absence of glucose. Right – AMPK activity of untreated WT and TSC1-/- MEFs maintained in complete growth medium supplemented with 10% serum. Notice the higher phosphoT172-AMPK levels (activity) due to the increased anabolic demand imposed by constitutive mTORC1 signalling in TSC1-/- MEFs. Bars are mean ± SD. Statistical significance was calculated using two-tailed t-Test. **p<0.01, **B)** Western blot of WT and TSC1-/- MEFs treated as indicated for 8h. Note that energy deprivation alone does not manifest as spontaneous DNA damage in TSC1-/- MEFs appear to initiate classical DNA damage response pathways – p53 activation and PARP cleavage. Also note the generally higher activation states of AMPK. GLc – Glucose, 2dG – 2-deoxy-Glucose, Torin1 – ATP competitive TOR Kinase inhibitor, L-Gln – L-Glutamine, Aa – Essential Amino acids, Nsd – Nucleosides.

5. Discussion

The serine/threonine kinase mTOR (mammalian target of rapamycin) within the insulin signalling pathway is a pivotal regulator of fundamental cellular aspects like cell size, transcription, translation, autophagy, cell survival and aging. Dysregulated mTOR, being at the centre-stage in the genesis of hereditary and lifestyle diseases including obesity, diabetes, degenerative disorders and cancer, has provoked intense interest from virtually all major therapeutic angles (Rosner & Hengstschlager, 2011). Over 80% of all human cancers acquire mTORC1 hyperactivity either directly or indirectly, and this has several transcriptional and translational consequences, all of which together drive uncontrolled growth and proliferation. The peculiar nature of tuberous sclerosis, compared to other cancers characterised by mTORC1 hyperactivity has been reviewed. Several groups have reported hypersensitivity to stress in tuberous sclerosis patients as well as in cellular and mouse models of the syndrome, addressed in detail in the introduction section. Cell cycle progression on the backdrop of high mTORC1 activity has also been addressed previously. However, whether these changes in cell cycle regulation influence stress response and its outcome in TSC1-/- cells has not been sufficiently addressed. We wished to study how exactly TSC-/- cells succumb to mild genotoxic stress resulting in cell death on the face of a robustly active growth signalling pathway. We have employed two widely used genotoxins, Hydroxyurea and Adriamycin. HU is a specific RRM2 (Ribonucleotide reductase M2) inhibitor that depletes intracellular nucleotide pools thereby causing replication blockade and stress, although the poor pharmacokinetic properties limit its clinical uses for cancer treatment. In vitro, acute HU-treatment arrests cells in S-phase and extended treatments are known to result in apoptosis. Adriamycin or doxorubicin is an anthracyclin intercalating chemotherapeutic first isolated from *Streptomyces peucetius* var. *causius* routinely used against several cancers including breast, lung, gastric, ovarian, thyroid, non-Hodgkin's and Hodgkin's lymphoma, multiple myeloma, sarcoma and paediatric cancers. The major side effect limiting its use is due to its cardiotoxicity. The mode of action of Adr is a) intercalation into DNA disrupting Topoisomerase-II-mediated DNA repair and b) conversion to an unstable semiquinone intermediate leading to free radical damage of DNA, proteins and membranes.

Our experiments revealed that TSC1-/- MEFs are generally hypersensitive to mild doses of both genotoxins used, although the effect of Adr on these cells was slightly more

pronounced. In attempts to understand the nature of cell death, MTT assays, AnnexinV labelling, and PI exclusion flow cytometry were performed. Although PI exclusion flow cytometry is an indicator of terminal death, other quantitative approaches resulted in gross underestimation of total death, for instance, the MTT formazan conversion assay, being a metabolic readout is an indirect indicator of cell viability. Considering that the entire apoptotic programme/cascade is executed in a short time-frame of an hour, our attempts to observe bona fide apoptotic death by AnnexinV labelling in WT vs TSC1-/- cells has been challenging, limiting its use in our setting. Additionally, caspase-3 cleavage assays and PARP-cleavage westerns have also been used as readouts for apoptosis. Our own blots of WT and TSC1-/- cells under genotoxic treatment (HU and Adr) showed that TSC1-/- MEFs not only have increased Caspase-3 and PARP cleavage, but also initiate caspase-3 cleavage at a much lower dose (Figure 15A) than their WT counterparts, implying that apoptotic dying accounts only for a fraction of the total non-viable cell population. PI exclusion assays projected a significantly higher overall death toll, suggesting there may be other non-apoptotic mild genotoxic stress-induced death programmes operating in TSC1-/- cells. One feature of TSC1-/- cells is the elevated levels of p53 expression, amenable to stabilisation and activation upon DNA damage or metabolic stress; and this has been linked to the elevated death toll in TSC1-/- cells (Lee et al., 2007). Super-threshold p53 expression levels are now understood to be an important factor tipping the balance towards apoptosis from a transient cell cycle arrest, largely by virtue of the dynamics of p53 promoter-binding to target cell cycle arrest vs pro-apoptotic gene sets (Aylon & Oren, 2007; Carvajal & Manfredi, 2013; Kracikova, Akiri, George, Sachidanandam, & Aaronson, 2013). We observe that mTOR inhibition with either rapamycin (allosteric inhibitor, mTOR complex1) or Torin1 (ATP-competitive inhibitor, complex1 and 2) provides relief on p53 stabilisation and activity, but confers virtually negligible protection against death as measured by PI exclusion assay. In view of the heterogeneity in the mode of action and effects of the genotoxic agents used, and the possible involvement of multiple death programmes, care should be taken in interpreting these results, also bearing in mind, p53-dependent and p53-independent cell death processes. We further ruled-out/excluded any secondary, misleading effects due to rewiring of signalling pathways and long-term adaptations of cell lines in culture, by acute siRNA-mediated knockdowns of TSC2 in WT MEFs; we could recapitulate signalling patterns

characteristic of TSC-/- cells both under resting and genotoxin-treated conditions (Figure 15B).

Whether the hypersensitivity to mild stress and death was an outcome of elevated primary genetic insults or an exaggerated damage response in TSC1-/- MEFs intrigued us. γH2A.x, the phosphorylated (S139) Histone variant H2A.x, is known to be activated in response to diverse stressors for e.g., drug-, free radical- or radiation-induced DNA damage (Dickey et al., 2010). It is an upstream sensor alarming DNA double strand breaks and can be visualised as discernible 'foci' since phosphorylated H2A.x spans grossly tens of thousands of kilobases on either side of a double strand break site so as to recruit DNA repair machinery. This property has been exploited as a means/marker to quantify primary DNA damage with specific antibodies on immunofluorescence or flow cytometry platforms as well as western blotting (Bonner et al., 2008; Mah et al., 2010; Pilch et al., 2003; Rakiman, Chinnadurai, Baraneedharan, Paul, & Venkatachalam, 2008). Here we observe that under mild genotoxic treatment, TSC1-/- MEFs accumulate higher γH2A.x levels, for short as well as extended periods of time, as long as the stress dose is maintained (0.5µg/mL Adriamycin). Interestingly, at lower doses, there was no apparent difference, whereas the prescribed dose of 0.5µg/mL Adriamycin appears to be a clear tipping point in the decision-making of TSC1-/- cells to initiate death programmes, since western blots indicate a sharp increase in γH2A.x phosphorylation and this correlates with the higher death count by PI exclusion assays. Such hormetic (non-linear) death outcome to low-dose stress has been a matter of intense investigation (Gems & Partridge, 2008; Mattson, 2008; Tsaponina & Chabes, 2013) in attempts to understand adaptive rewiring of signalling circuitry and is the central theme of the RTG1715-adaptive stress response graduate research programme.

One cellular manifestation of oncogene activation is alterations in cell cycle distribution. Several oncogenes force G1/S transition and drive rapid proliferation. mTORC1 is popular for its function in cell mass and growth control. Constitutive mTORC1 activity has been shown previously to truncate G1 phase and prolong S-phase in patient material (T. Soucek et al., 1998; Wataya-Kaneda et al., 2001). Our studies of TSC1-/- MEFs revealed an increased proportion of S-phase cells accompanied by a compensatory/concomitant decrease in G1 cells. mTORC1 controls cell proliferation by a) anabolic regulation of protein synthesis and cell mass and b) transcriptional regulation of G1/S phase cyclin(s) expression described in

more detail earlier. Consistent with their timing of activation, Cyclin E-Cdk2 and Cyclin A-Cdk2 complexes have pivotal functions in DNA replication initiation and progression respectively. Cyclin E-Cdk2 controls replication initiation a. by releasing E2F from Rb driving S-phase specific gene expression and b. by phosphorylating and regulating components of the pre-replication complex at the time of Origin licencing. Cyclin A-Cdk2 controls DNA synthesis by stimulating function of the pre-replication complex and activating replicative DNA polymerases, while at the same time preventing re-replication by prohibiting re-firing of used origins in which Mcm4, a component of the CMG helicase complex is phosphorylated and inhibited (Woo & Poon, 2003). While Cdk2 activity in S-phase control appears to be redundant, owing to the capacity of other Cdks to compensate for its loss, Cdk2 function in DNA damage response seems non-redundant. Although Cdk2 activity is abolished following DNA damage, and its inhibition plays a crucial role in DNA damage-induced cell cycle arrest and repair, its direct role, if any, in repair processes and cell death ensuing irreparable damage has been questioned. A central role for Cdk2 in several DNA damage response and repair processes is emerging (Satyanarayana & Kaldis, 2009). For instance, Cdk2 inhibition has been shown to delay damage signalling via Chk1, p53 and Rad51 and also hinder DNA repair in checkpoint and repair deficient cells, particularly BRCA1 and ATM-mutant tumour cells. Other important constituents of the double strand break repair machinery including Ku70 and the Nijmegen Breakage Syndrome gene product Nbs1, a component of the conserved Mre11-Rad50-Nbs1 (MRN) complex, are activated after DNA damage in a Cdk2-dependent manner (Müller-Tidow et al., 2004; Wohlbold et al., 2012). Furthermore, Foxo1 phosphorylation by Cdk2 and cytoplasmic retention was abrogated in DNA damage-induced apoptosis, implying a role for Cdk2 in cell survival (Huang, Regan, Lou, Chen, & Tindall, 2006). To our surprise, in addition to the previously described p53 overabundance, we have also observed Cdk2 downregulation in TSC1-/- MEFs (Figure 18B). It is conceivable that constitutive mTORC1 activity and the augmented protein synthesis (cell mass build-up) ‘misleads’ cells into premature S-phase entry. In fact, CFSE dye dilution-based doubling time measurements and estimation of cell cycle phase lengths (data not shown) indicated a 50% reduction in G1 length and a1.5-fold increase in S-phase length. Moreover, the crosstalks of Cdk2 with the S-phase checkpoint components and its unique functions in double strand break repair may be compromised in TSC1-/- cells contributing to its sensitivity.

One major aim of the project has been to decipher the mechanisms by which mTORC1 fulfils its sensitizing function, resulting in loss of mild-stress adaptation following loss of TSC function. DNA replication in S-phase is the most vulnerable process that may lead to the accumulation of mutations, genetic alterations and chromosome instability (Osborn et al., 2002). Hence, rapid proliferation (shorter doubling times) and a lengthy S-phase apparently predispose TSC1-/- cells to DNA damage. Replication stress (RS) is an immediate consequence of oncogene activation, Ras, myc, and Cyclin E being the well-described examples (Toledo, Murga, & Fernandez-Capetillo, 2011). Although how exactly they cause RS remains an enigma, inappropriate use (over- or under-firing) of replication origins appears to be the modus operandi of oncogenes known to cause RS (Abulaiti, Fikaris, Tsygankova, & Meinkoth, 2006; Gaillard et al., 2015; R. M. Jones et al., 2013; Maya-Mendoza et al., 2015; Rohban & Campaner, 2015).

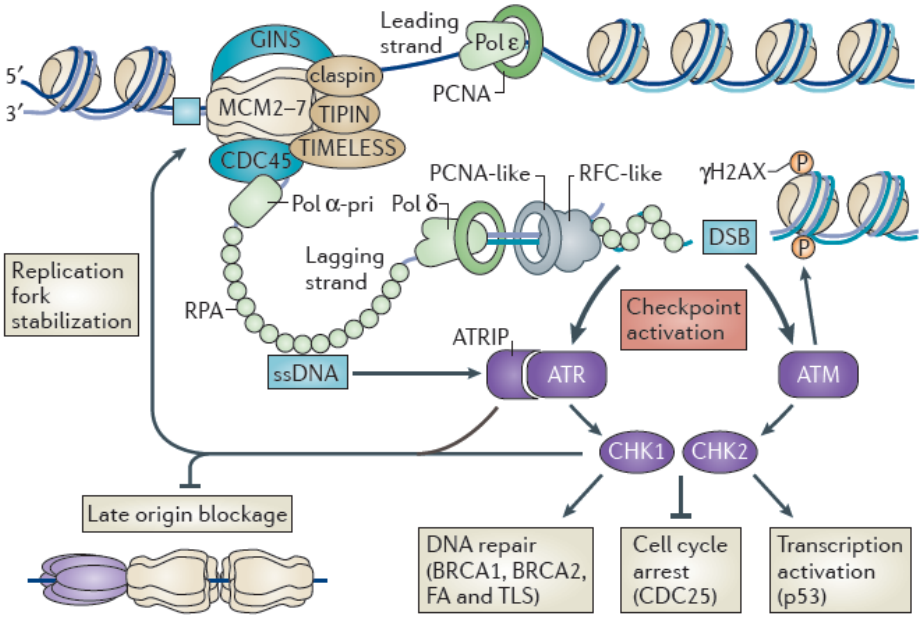


Figure 22: The replication stress response.
The figure depicts the execution and functions of the replication checkpoint ensuring faithful duplication of genetic material in the S-phase. Replication impedances are a common occurrence. The kinases ATR and Chk1 coordinate a genome surveillance system that includes fork stabilisation and restart, suppression of late origin firing, cell cycle arrest and activation of transcriptional programmes, all of which allow cells to cope with replication stress. Figure reproduced with permission from Gaillard H., *et. al.,* 2015, nature reviews, 15; pp.279.

ATR and Chk1 kinases, as part of the replication checkpoint, play a crucial role in coordinating a complex network of surveillance processes that together ensure faithful duplication of DNA in the S-phase (Joaquin & Fernandez-Capetillo, 2012; Lopez-Contreras, Gutierrez-Martinez, Specks, Rodrigo-Perez, & Fernandez-Capetillo, 2012). Although ATR haploinsufficiency is associated with a small increase in tumour incidence (Eric J. Brown & Baltimore, 2000), tumour cells with high replication stress often upregulate ATR and Chk1 levels, so as to combat deleterious RS consequences, while maintaining adequate RS checkpoint activity to evade cell cycle arrest and promote cell survival. The critical dependence on the ATR-Chk1 pathway to alleviate RS in cancers is being intensively studied under various settings as a therapy for cancer (Toledo et al., 2011). ATR upregulation is often synthetic-lethal with p53 function, for which reason, most tumour cells undergo selection for loss of p53 function (Lecona & Fernandez-Capetillo, 2014) and ATR downregulation is synthetic lethal with oncogenic Ras under certain contexts (Gilad et al., 2010). Chk1 functions on the other hand to inhibit late origin firing, stabilise and re-start stalled forks and its loss is known to drastically impede fork progression rates (Ge & Blow, 2010; Petermann et al., 2006). We observe a down-regulation of ATR kinase levels, probably indicative of the complex transcriptional programme controlled by high levels of functional p53 in TSC1-/- cells. Reports claiming ATR may modulate nucleotide metabolic pathways to buffer replication stress (Lecona & Fernandez-Capetillo, 2014) suggest that the HU hypersensitivity of TSC1-/- MEFs may well be a consequence of ATR downregulation. ATR also plays an important role in coordinating chromosome condensation with nuclear envelope breakdown (Lecona & Fernandez-Capetillo, 2014), raising the possibility that the G2-M checkpoint override we observe, and mitotic catastrophe per se, may be influenced by ATR downregulation.

Persistently high nucleotide incorporation rate is the first sign of RS *in vitro*, and owing to the diverse sources of RS, its markers activation of the ATR pathway and include γH2AX (Ser139), RPA (phospho Ser33), Chk1 (phospho Ser345), detection of single stranded DNA directly by native BrdU immunofluorescence or indirectly by RPA foci formation. However, the most reliable readout of RS is the direct measurement of DNA replication by means of the DNA fibre or molecular combing assays (Jackson & Pombo, 1998; Zeman & Cimprich, 2014). We have observed that while untreated TSC1-/- MEFs over-fired modesty replication origins decreasing inter-origin distance marginally compared to WT MEFs, they were all 1st pulse

(CldU), excluding the misuse of dormant origins. In addition, even though replication forks progressed at a slower rate in TSC1-/- MEFs, bidirectional progression was rather 'clean' with no spontaneous asymmetry, a striking deviation from classical RS. Furthermore, a dramatically elevated fork asymmetry upon acute Adriamycin treatment supports the hypothesis that TSC1-/- MEFs accumulate excessive DNA damage as seen from the γH2A.x measurements. Together, these results prove that TSC1-/- MEFs have an increased propensity to gather genetic insults from genotoxic agents. The decline in fork progression rates along with the lack of asymmetry led us to interpret and propose an ATP-restricted replication phenotype driven by constitutive mTORC1 activity, distinct from canonical RS. We propose that such ATP-restricted replication is a reflection of low-energy atmosphere in TSC-/- cells accompanied by the marginal origin over-firing, thus affecting replicative helicase function within the CMG initiation complex. In fact, the downregulation of cdc45 with adriamycin treatment (together with the down-regulation of Cdk2) argues for instability in initiation complex formation also suggesting that cellular energy is exceedingly diverted for ATP-requiring repair processes, slowing down fork speed.

Significant crosstalk between AMPK, mTOR and p53 in a signalling triad, to match nutrient sufficiency, with growth signalling and stress, has been described (Feng et al., 2007). AMPK alone is sufficient to cause a reversible cell cycle arrest upon nutrient (glucose) deprivation via both mTORC1 inhibition and p53 (Ser15) priming phosphorylation, in a G1/S metabolic checkpoint, and prolonged AMPK activity drives cellular senescence, not apoptosis, in normal MEFs. Interestingly, such a metabolic checkpoint is effective in spite of continued amino acid availability and sufficiency. When cells are transformed however, with active oncogenes, such an AMPK response leads to stabilisation following p53 Ser15 phosphorylation and apoptotic death. Thus AMPK is able to execute a p53-dependent apoptotic response to metabolic stress in several tumour cells experiencing sustained growth signalling (R. G. Jones et al., 2005). Two particular genotoxic stress-induced p53 target gene products namely Sestrin1 and Sestrin2 were recently described to relay signals from active p53 to AMPK, thus activating it. This in turn activated TSC1/2 complex resulting in the shut-down of mTORC1, providing a novel link between mTORC1 pathway and p53 under genotoxic stress. Moreover, it was shown that Sestrins physically interact with TSC1, TSC2, and AMPK so as to exert their inhibitory effect (Budanov & Karin, 2008). However, in the absence of a functional TSC complex (TSC1-/- or TSC2-/- cells), other factors contributing

to their stress sensitivity gain priority, although AMPK inhibits mTORC1 by Raptor phosphorylation (Gwinn et al., 2008). AMPK-mediated apoptosis under metabolic and genotoxic stress in mTORC1 hyperactive tumour cells has been documented (Lee et al., 2007), but mTORC1 hyperactivity seems to predispose cells to stress-induced death independent of p53 (Choo et al., 2010). TSC-/- cells are known to have higher AMPK activity. However, whether the actual deficit in energy or the signalling via AMPK influences the outcome of mild genotoxic stress on TSC-/- cells is not fully understood. Here, we have briefly addressed this issue by objectively manipulating three factors, energy supply, mTORC1 activity and Replication-phenotype alleviation, all in presence or absence of mild genotoxic stress. It appears that L-Gln and Nsd supplementation prevents or rescues damage accumulation, while maintaining cellular energy levels (γH2A.x and p-T172 AMPK levels, Figure 21).

To summarize, TSC1-/- MEFs are hypersensitive to mild genotoxic stress due to their cell cycle irregularities and partly due to elevated p53 expression levels, and is exacerbated by their poor energetic milieu, although apoptotic and non-apoptotic modes of dying may cooperate.

Table 10: Summary of features of untransformed, oncogene-driven and mTORC1 driven tumour cells

Feature		Untransformed (Normal) cells	Classical Replication Stress Ras-, Myc-, Cyclin E-driven tumours	ATP-restricted replication phenotype Tuberous Sclerosis Complex
Growth Signalling	mTORC1 status	+	++	+++
	p53 status	+	- (usually lost)	+++ (upregulated)
Replication properties	Origin firing	+	+++ (gross over-use)	++ (modest over-firing)
	Ori-Ori distance	++++	++ (drastic reduction)	+++ (marginal reduction)
	Fork progression	++++	+++	++
	Fork asymmetry	-	+++ (excess stalling)	- (absent)
	Origin re-firing	-	+	-
	Nucleotide pool	++	+	?
	ATP levels	+++	++	++
RS Checkpoint proficiency and response	ATR expression	++	++++ (upregulated)	+ (down regulated)
	Chk1 expression	++	++++ (upregulated)	++ (unchanged)
	Under-replicated DNA	-	+++	- (virtually absent)
Cellular outcome	Chromosome breaks, rearrangements and instability	-	+++	-
	Stress sensitivity	+	- (evade apoptosis)	+++ (hypersensitive)
Tumour properties	Invasive Capacity	-	+++ (malignant, aggressive)	- (benign, hamartomas)

Although our findings from cell cycle analysis and DNA fibre assays are strongly suggestive of an ATP-dependent slump in synthesis rates in TSC1-/- cells, we are currently in pursuit of DNA fibre experiments performed with the sole intent of confirming whether or not, feeding TSC1-/- MEFs with high-energy substrates (L-Gln, Nsd, along with appropriate controls) can restore synthesis rates to near-WT rates, compared to TSC1-/- cells placed under standard growth conditions. These results are eagerly awaited, but unfortunately not be available in good time to be part of this thesis. However, these findings would serve to evidence and support several previous observations, provide a fundamental explanation to the same, as well as pave the way for conceptualising novel therapies for TSC. For instance, based on the fact that TSC-/- cells accrue higher genetic insult and respond inappropriately, confirmatory data proving the ATP- restricted DNA synthesis may collectively be exploited as a basis to design targeted therapies for selective killing of TSC tumours by low-dose chemotherapy and

calorie-restricted, amino acid-rich adjuvant nutritional therapies. Further understanding of the precise nature of replication control in cells with high mTORC1 activity harbouring diverse mutations in TSC1/TSC2 alleles will facilitate translational approaches to TSC therapy. We have, in this piece of work, provided evidence of the previously reported lengthy S-phase of TSC-/- patient material and put-forth, a novel molecular biological explanation as to why TSC tumours remain benign.

Based on our data sets, we conclude that inherent defects in their cell cycle regulation render TSC1-/- cells supersensitive to mild genotoxic stress. The moderate over-firing of replication origins escalates demand for energy and building blocks; a slower fork progression rate within a longer S-phase 'exposes' nascent DNA making it vulnerable to damage accumulation; constitutive mTORC1 activity by way of p53 upregulation and ATR downregulation provides resilience to cell cycle arrest and overrides/dampens checkpoint responses, all together under mild external genotoxic stress and low intrinsic ATP availability proves detrimental to their survival. It is noteworthy, that such sensitivity, by eliminating genetically compromised cells resulting from excessive spontaneous damage in TSC tumours may explain their benign property. This revelation of an "Achilles' heel" for TSC1-/- cells may be further explored to harness the therapeutic potential of low-dose chemotherapy and calorie-restricted supplementary care as treatment options for tuberous sclerosis.

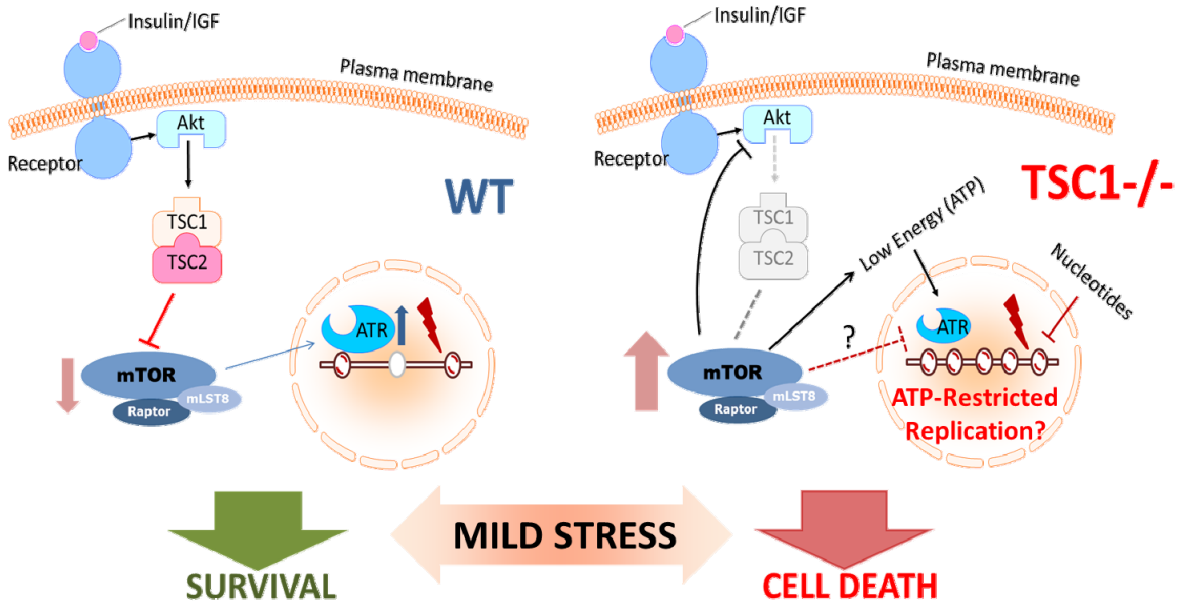


Figure 23: Model summarising various factors converging in the loss of mild stress adaptation in TSC1-/-cells.

A constitutively anabolic state with increased energy expenditure, perturbed cell cycle progression including S-phase checkpoint kinase–ATR downregulation, a restrained replication phenotype with modest origin over-use and declined fork progression rates, high p53 levels, altogether set stage for a failure of adaptation of TSC1-/- cells to mild external stress doses, given the inherent stress milieu.

6. Conclusions and Future Directions

Tremendous progress has been made in the understanding of genetics and pathophysiologic aspects of this serious and multi-system disorder. A multidisciplinary approach for an early, accurate diagnosis and further insight into the genotype-phenotype relationships in TSC is essential for proper management of affected individuals. The delineation of the TSC biochemical signalling pathways have emerged as strategies for developing mTOR inhibitor therapies, currently under clinical trials. Our data suggest that constitutive mTORC1 activity, by promoting a non-canonical ATP-restricted replication phenotype, causing energetic debility and DNA damage checkpoint override, proves detrimental to cellular stress adaptation. Importantly, our interpretation of the spontaneous asymmetry-free S-phase, a deviation from classical RS, may provide novel insight as to why TSC tumours are detained in a benign state and opens avenues for low-dose chemotherapy-based therapeutic intervention in TSC patients. We hypothesise that the observed slack in DNA synthesis is primarily a result of energy/ATP shortage, although there is no compelling evidence arguing for nucleotide sufficiency or dysregulation of other factors influencing DNA replication at this stage. Further experiments in this direction may causally tie energetic compromise in TSC1-/- cells to the previously observed lengthy S-phase in TSC patients while offering an explanation as to why TSC tumours are detained in a benign state, although multi-organ involvement affects quality of patients' life. Insights into the role of hyperactive mTORC1 in cell cycle progression may open a paradigm for targeted therapies aimed at selective killing of TSC tumours, taking advantage of a) the ATP-restricted replication phenotype and the associated hypersensitivity to otherwise harmless chemotherapeutic doses, also overcoming rapamycin-refractory tumours/cases and b) possibly for developing an adjuvant nutritional (low glucose, high amino acid-based) therapy regimen.

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Pai MG. Cytogenetic monitoring of human lymphocytes for drug action employing interphase condensed DNA. Proc. of Indo-Aus. Symposium on pharmacogenomics; 10th Mar 2006; Manipal, India. p.16.

LABORATORY SKILLS / TECHNIQUES

Animal Cell Culture and cell biology

Mammalian Cell Cultures (hPBLs, CHO, Vero, HeLa, MEF and sp2/0 cell lines), Sphere cultures and hanging-drop cultures, Cell proliferation and viability assays, multi-colour flow cytometry.

Molecular biology and Biochemistry

DNA and RNA isolation, Polymerase Chain Reaction and gene cloning, Agarose and Polyacrylamide gel electrophoresis of DNA, SDS-PAGE for proteins, Transfections and gene silencing, Western blotting.

IPTG-inducible prokaryotic (bacterial) expression systems, Chromatographic protein purification.

Human Cytogenetics

Standard metaphase karyotyping, banding techniques and chromosome analysis, In situ hybridization.

Immunology

Basic Immunotechniques of Antigen-Antibody interactions, diffusion-based and immunoelectrophoresis.

Rabbit polyclonal antibody generation and titre studies. General animal handling (mice).

OTHER SKILLS

IT Proficiency – MS word, MS Excel, MS PowerPoint, Adobe Photoshop and Illustrator. Basic Bioinformatics.

Driving Licence – two and four wheeler license (India)

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AWARDS AND STANDARDISED TESTS

Qualified GATE Life Sciences (Feb 2006).

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GRE general test, March 2007 [score – 1150 + writing band 4]

GRE Subject test, 13th November 2010.

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INTERESTS & ACTIVITIES

Playing cricket, Swimming, Traveling, Cooking, Indian Philosophy and Ayurveda.

Actively participated in intercollegiate quiz contests at the undergraduate and post-graduate level.

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Ehrenwörtliche Erklärung

Hiermit erkläre ich ehrenwörtlich im Zusammenhang mit der Beantragung der Eröffnung meines Promotionsverfahrens:

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